THE DEVELOPMENT OF A SILICONE RUBBER SURFACE INHIBITORY TO THE ADHERENCE OF MICROORGANISMS

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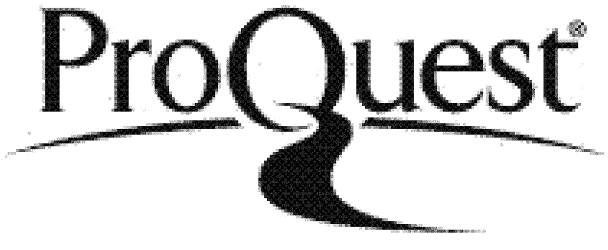
Submitted for the degree of Doctor of Philosophy University of Wales
June 2004



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ACKNOWLEDGEMENTS

Thanks especially to my wonderful principal supervisors David Williams and Mark Waters, who have been fantastic and without whom I would never have finished!

I would also like to thank my supervisor Mike Lewis and also Melanie Wilson, who helped me with the saliva collection and Rachel Waddington for her expert advice on protein purification.

I would also like to thank my supervisor David Stickler and the rest of his group at Cardiff University including Nicola Morris, Steve Jones and Nora Sabubba for help and advice on the bacterial work in this thesis.

Thanks to Lisa Coulthwaite from Manchester Metropolitan University for performing microbiological assays on my treated silicone rubber and to Henk Busscher's group at Gronningen University in Holland for performing the artificial throat assays.

This thesis would never have been completed without the help and support of my friends, family and colleagues. I am grateful that I had Helen Roberts and Kath Leach studying for a PhD at the same time so we could talk through our problems and celebrate when our work was going well. Special thanks go to Helen who provided me with valuable help with my protein work. Thanks also to my parents who provided emotional support throughout.

Finally, I have to mention Harvey who has listened to every single problem, shared every success and also witnessed the odd tantrum! Harvey this thesis is dedicated to you.

SUMMARY

Silicone rubber is a widely used biomaterial but problems arise when microorganisms colonise its surface forming a biofilm. Voice box prostheses and denture soft liners are medical devices which are susceptible to *Candida* colonisation, whilst urinary catheters are prone to colonisation with *Proteus mirabilis.* Microbial colonisation can lead to clinical infection and device dysfunction. In this study, 8 surface-modified silicone rubbers were developed (T1-T8) with the aim of preventing initial microbial adherence. The surfaces were activated using argon-plasma discharge treatment, which was followed by silane coupling. The resulting surfaces had varied surface functional group chain length, chemistry and mobility. *Candida albicans* and *P. mirabilis* adherence assays were performed in the presence and absence of salivary and urinary conditioning films. Results demonstrated a significant decrease in adherence on all the treated surfaces compared to the control. The material most successful at inhibiting candidal adherence was T5 (reduced adherence 50-fold, without saliva present) and most successful at inhibiting *P. mirabilis* was T1 (reduced adherence by half, without urine present). This surface also reduced the motility of *P. mirabilis* by 80%.

It was concluded that reduced adherence was due to the increased mobility of chemical groups present on the modified material surfaces.

In the presence of salivary and urinary conditioning films, adherence increased, although several of the saliva-coated surfaces maintained lower candidal adherence levels than the control. These lower adherence levels could not be attributed to lowered adsorption of the *Candida*-binding proline-rich proteins to the treated material, as hypothesised.

A second aspect to the study involved determining the surface roughness of a variety of commercially available urinary catheters and relating their roughness to the motility rates of *P. mirabilis.* Motility rate was highest on the roughest catheter surfaces (hydrogel and silver/hydrogel) suggesting that manufacturers should consider topographical features as well as antimicrobial coatings when attempting to combat microbial colonisation.

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ABBREVIATIONS

ANOVA	Analysis of variance
AFM	Atomic force microscope
ASTM	American society for testing materials
CAC	Chronic atrophic candidosis
CAUTI	Catheter-associated urinary tract infection
CFU	Colony forming units
eV	Electron Volts
FC	Flow cell
FN	Fibronectin
FT-IR	Fourier Transform-Infrared Spectroscopy
h	hours
kDa	kilodaltons
KN	Kilonewtons
mbar	millibar
N	Newtons
NCYC	National collection of yeast cultures
nM	nanomolar
PBS	Phosphate buffered saline
PEO	Polyethylene oxide
PDMS	Poly(dimethylsilaoxane)
PRPs	Proline-rich proteins
Ra	Average roughness
RU	Resonance units
Rz	Z-range
s	seconds
SD	Standard deviation
SEM	Scanning electron microscope
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UTI	Urinary tract infection
V	Volts
W	Watts
XPS	X-ray photoelectron spectroscopy
YNB	Yeast nitrogen base
λ_{em}	Wavelength emission intensity
λ_{ex}	Wavelength excitation intensity
µl	microlitres
µm	micrometers

Chapter 1

Introduction

1.1 Biomaterials

Biomaterials are materials used within a biological setting and can be described as any substance, natural or synthetic, which at some stage, interfaces with tissue (Mardis *et al.*, 1988). Their use is necessary when physiological systems dysfunction or body parts are lost due to trauma or congenital defects. Biomaterials amend dysfunctions which demand physical assistance rather than medication or other therapies. Biomaterials need to have certain physico-chemical properties to prevent or reduce the likelihood of alterations or damage to biological systems. Crucially, the materials must be non-toxic, inert and durable.

The use of biomaterials dates back to ancient times, with descriptions of Egyptians using lead and papyrus catheters for urinary drainage (Bitschai and Brodny, 1956). During the 16th Century a French military surgeon named Ambroise Pare described methods of rectifying facial defects by means of facial prostheses. These rudimentary prostheses were made from papier-mâché, leather, ivory, gold and silver (Conroy, 1993). The earliest implantable prosthetic devices were designed and used for replacing damaged or diseased parts of the urinary tract. Ureteral replacement using glass, tantalum or vitallium tubes has been described, but success was limited by distal migration of the prosthesis out of the ureter (Denstedt *et al.*, 1998). It was not until 1901 that Upman, an American dentist reported the medical use of more modern and practical materials such as vulcanite rubber to fabricate oral and nasal prostheses (Upham, 1901).

One of the most important uses of biomaterials is in the manufacture of medical devices. Medical devices can be used both internally and externally to the human body and aid in both patient treatment and function. Each year, billions of medical devices are used in clinical practice (Reid, 1994). Examples of medical devices include: catheters, artificial skin, artificial blood vessels, artificial heart valves, pacemakers, dental fillings, oral prostheses, artificial voice boxes, wire plates and pins for bone repair and total artificial joint replacements.

The chemical composition of biomaterials varies depending on their function. For instance, hard metals such as amalgam are used for filling tooth cavities, high-strength, lightweight materials such as titanium are used for joint replacements and soft flexible polymers such as silicone rubber are ideal for the easy insertion and comfort of urinary catheters. The impact of biomaterials on the improvement of patient life ranges from visionary improvement through the use of contact lenses to the total life changing effects from the receipt of artificial heart valves.

Biomaterials used in the oral cavity include acrylic as a material for dentures and denture soft liners, ceramic for veneers and crowns, amalgam and gold for tooth fillings and titanium for root implants. Silicone rubber is often used for the production of denture-soft liners and voice box prostheses. Denture-soft liners provide a cushion between the palate and the denture acrylic, which reduces frictional irritation. Voice box prostheses are implanted in patients who have undergone a total laryngectomy and these devices enable speech formation. These devices are discussed in greater detail in Section 1.2.1.

1.2 Silicone rubber medical devices

Medical devices are commonly made from silicone rubber (Figure 1.1) due to its excellent physical and chemical properties (Table 1.1). Silicone rubber polymers are large molecules comprised of thousands of monomeric units, which are linked by polymerisation. The chain structure is similar to that of the hydrocarbons with silicon replacing carbon. There are different types of silicone rubber, the simplest being poly(dimethylsiloxane) (PDMS) which provides the basis for many medical devices.

The first silicone polymer was developed by Ladenburg in 1870s by reacting diethoxy diethyl silane with water in the presence of acid. Following this pioneering work, the PDMS polymers that are commercially available today were produced by reacting silicon with methyl chloride.

Silicone reacting with methyl chloride to produce dimethyl dichloro silane.

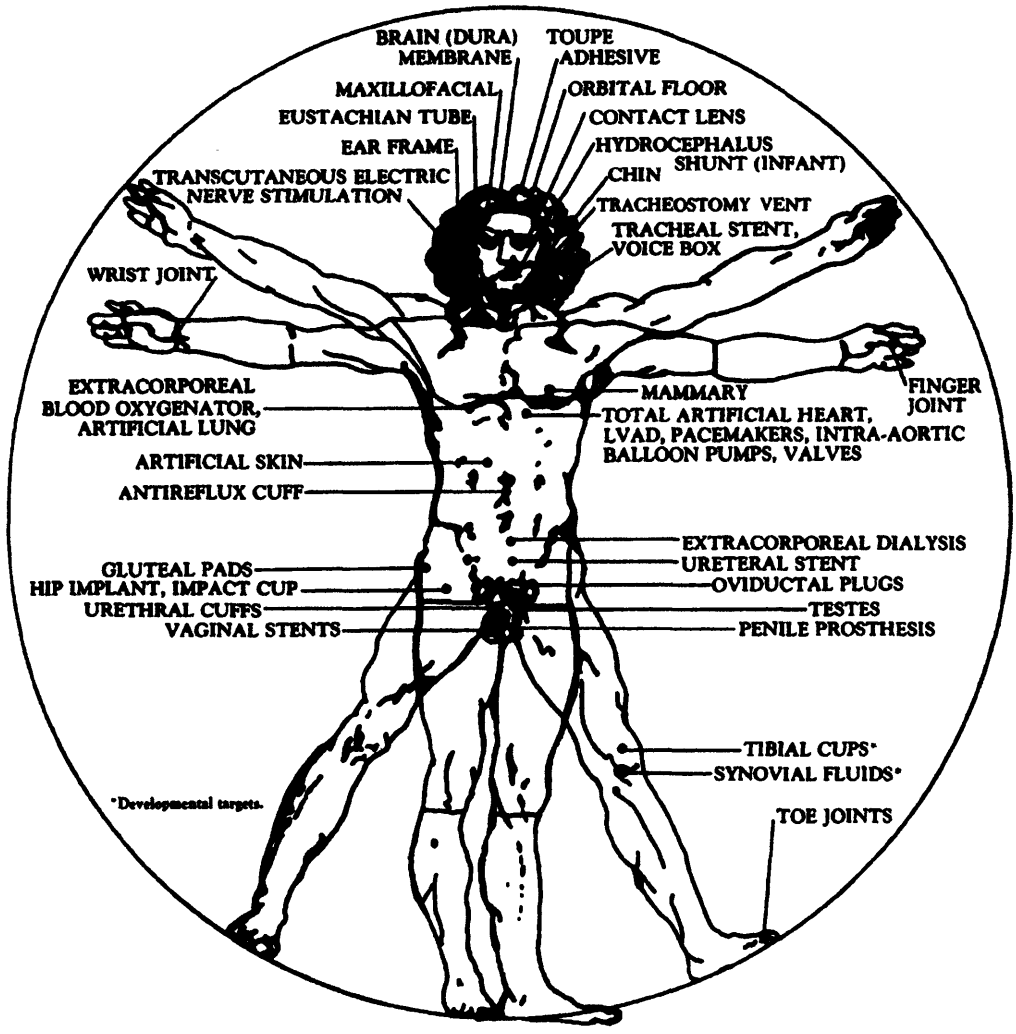
$$Si + 2\,CH_3Cl \longrightarrow (CH_3)_2SiCl_2$$

The resulting dimethyl dichloro silane was then hydrolysed to form the PDMS polymer (Williams, 1990).

Dimethyl dichloro silane is hydrolysed to form a PDMS.

$$(CH_3)_2SiCl_2 \xrightarrow{H_2O} (CH_3)_2SiO$$

Figure 1.1: Diagram illustrating the diversity of medical devices made from silicone polymers.



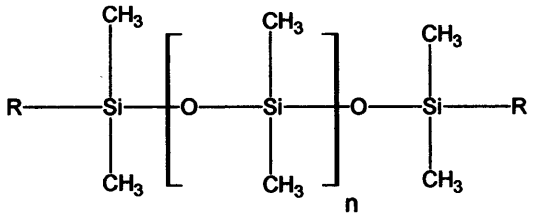
Taken from Arkles, (1987)

Table 1.1: Properties of silicone rubber that promote its use in medical devices

Property	**Advantages**
Inert	Will not react or break down
Non-toxic	Not harmful and non allergenic
Tasteless	Important when utilised in the oral cavity
Hard-wearing	Vital for long-term durability
Flexible/pliable	Allows easy insertion into veins etc.
Easy to manipulate	Easy material to manufacture and mould
Low cost	Cost effective

The degree of polymerisation (number of 'n' units) can vary, with longer chains producing high viscosity and elastic properties and shorter chains producing an elastomer with a low molecular weight, low viscosity and plastic or rigid properties.

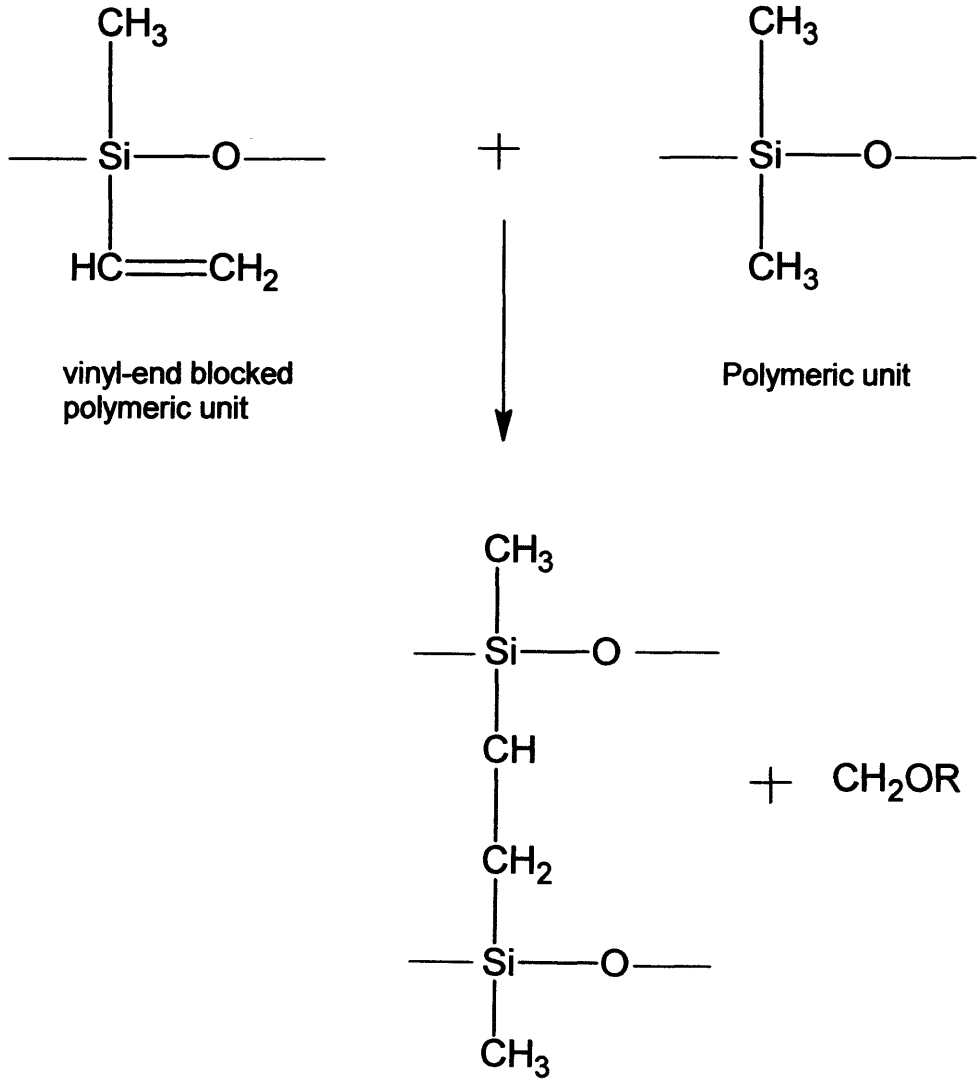
Dimethylsiloxane repeat unit



R = the end unit, which can include hydroxyl, methyl or vinyl groups

PDMS can be end blocked with hydroxyl (OH), methyl (CH_3) or vinyl (CH=CH) groups, which allow the PDMS chains to cross-link into a three dimensional network. This process is termed curing or vulcanisation, the result of which produces a solid silicone elastomer. There are several different curing methods including condensation, peroxide and addition reactions. Condensation reactions cure short-chained hydroxyl end-blocked PDMS polymers at room temperature, using polyfunctional silanes as cross-linking agents together with catalysts such as dicarboxylate tin. When all three are mixed together, curing takes place through silanol-alkoxysilane condensation resulting in the elimination of an alcohol. The peroxide curing system involves the heating of poly(organosiloxanes) with small amounts of organic peroxides in a polyaddition reaction. Free radicals arising due to the presence of peroxides are able to extract hydrogen atoms from methyl groups and ethylene links can thus be formed between siloxane bonds. The addition curing system involves the cross linking of a vinyl-end blocked (-CH=CH_2) PDMS and a hydride functional siloxane co-polymer in the presence of a platinum catalyst (Figure 1.2).

Figure 1.2: Addition curing of poly(dimethylsiloxane).



The process can be accelerated at a higher temperature if a heat activated platinum catalyst is used. Silica filler (SiO_2) is added to silicone polymers to increase tensile and tear strength, and abrasion resistance (Arkles, 1983). The average diameter of a silica particle is 7 - 40 nm and the property conferred through its addition to the silicone polymer is enhanced durability. The filler provides reinforcement by creating multifunctional bridges between the polymer chains which allow stress to be transmitted more uniformly when the material is under load. Silica fillers can be surface treated in order to alter their hydrophobicity. Hydrophilic silicas usually have hydroxyl groups incorporated on to their surface which can be replaced by methyl groups to produce hydrophobic silicas. The hydrophobicity of the silica filler can influence the overall properties of the elastomer, including the mechanical strength and the extent of water absorption. The surface of the silicone elastomer can also be modified by altering the surface chemistry. This alteration can be performed using silanes which contain functional groups that become incorporated on to the silicone surface via siloxane bonds. Due to the inert nature of silicone rubber, an activation process precedes silane treatment which usually involves plasma discharge treatment. This procedure makes the rubber surface reactive to allow the functional groups within the silane to become bonded. Silane surface treatments are employed to give the silicone surface different properties which can improve its performance. Often, silanes are used to alter the hydrophobicity of a surface but more specifically can be used on glassware to reduce the adsorption of red blood cells or enhance the adsorption or bonding of specific proteins such as heparin (Arkles, 1987).

The true potential of silicone rubber for medical applications was discovered when the material was used as the basis of a hydrocephalus shunt (Denstedt *et al.,* 1998).

Following this success, the medical uses of silicone rubber have expanded and now include its use in prosthetic heart valves, tissue expanders, finger joints and drug delivery systems. An important feature of silicone rubber is its flexibility which makes it particularly useful for devices such as intravenous and urinary catheters, artificial skin, contact lenses and intra-aortic balloon pumps. For these, and many other devices, silicone rubber is probably the best material that can be utilised.

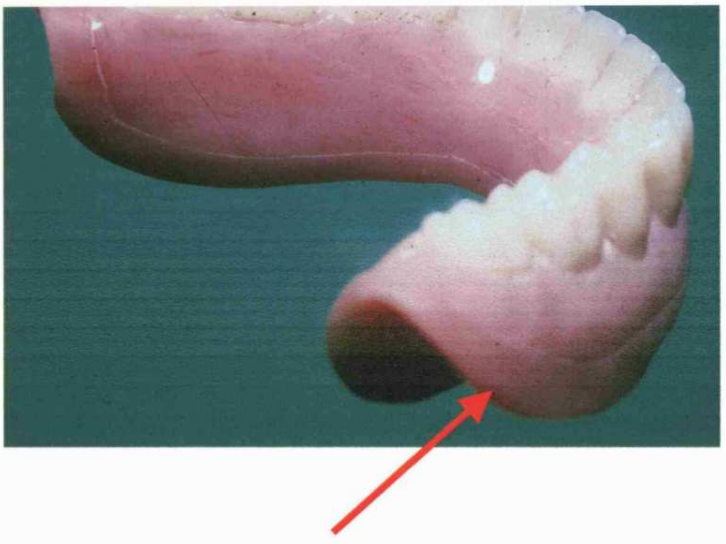
1.2.1 Denture-soft liners

Silicone rubber denture-soft lining materials are applied to the surfaces of dentures to reduce trauma to the soft supporting tissues. The liners are most commonly used beneath the lower denture and operate by creating an even distribution of functional loads over the denture bearing area, avoiding local concentrations of stress (Wright, 1980; Figure 1.3).

1.2.2 Voice box prostheses

A voice box prosthesis is a device fitted following a total laryngectomy. A total laryngectomy involves the removal of the larynx from the throat and is performed as part of the surgical treatment for laryngeal cancer. Laryngectomies are undertaken primarily in advanced stages of the disease and when radiation therapy fails. The procedure not only leads to the loss of natural speech, but is very traumatic for the patient, and presents a lifelong challenge for communication (Blom *et al.*, 1998). To restore speech several options are available. The tracheosophageal puncture technique introduced in 1979, involves the use of an indwelling voice box prosthesis,

Figure 1.3: Denture-soft liner attached to denture base.



which is positioned between the trachea and oesophagus. The prosthesis is a shunt valve, usually made from silicone rubber and allows patients to produce tracheo-esophageal speech. It is estimated that 80% of tracheoesophageal puncture patients achieve fluent, intelligible speech (Blom *et al.*, 1998). The prosthesis functions using a one way valve to allow sufficient shunting of expiratory air to the oesophagus without leakage of oesophageal contents into the trachea (Figures 1.4 and 1.5).

1.2.3 Urinary catheters

The genito-urinary tract is an anatomical region where a range of medical devices are utilised. Ureteral stents provide a passage for urine from the kidney to the bladder where blockages or anatomical irregularities are apparent. The urinary catheter (Figure 1.6) delivers urine from the bladder to a urine collection bag. The use of urinary catheters is very common, particularly in elderly and long-term hospitalised patients (Maki and Tambyah, 2001; Newman, 1998). Silicone rubber is ideal as a basis for urinary catheters as it is soft and flexible, thereby promoting easy insertion. The most common form of urinary catheter is the Foley catheter, which has an inflatable balloon towards the catheter tip which ensures the catheter is retained in position in the bladder. Once fitted, the end of the catheter is connected to a drainage tube, which is in turn attached to a urine collection bag. Listed below are the clinical uses of urinary catheters.

- Relief of urine retention caused by anatomical or neurophysiological blockage of the urethra

Figures 1.4a and 1.4b: Photographs of a 'Groningen Button' voice box prosthesis. *Fig.* 1.4a shows the voice box *in vivo* and *fig.* 1.4b displays an explanted prosthesis with biofilm apparent upon the surface. Images from Eerenstein *et al.*, (1999).

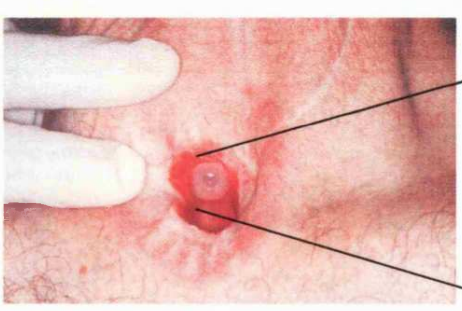


Figure 1.4a

Figure 1.4b

Figure 1.5: Schematic diagram of the silicone rubber 'Groningen Button' voice box prosthesis.

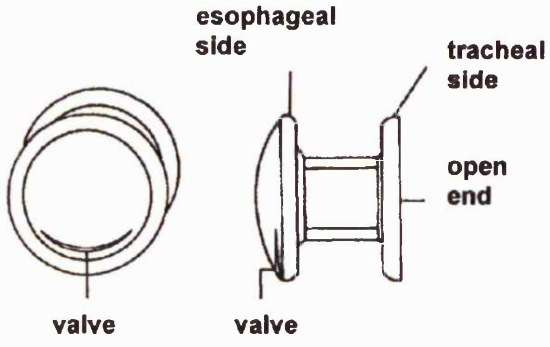
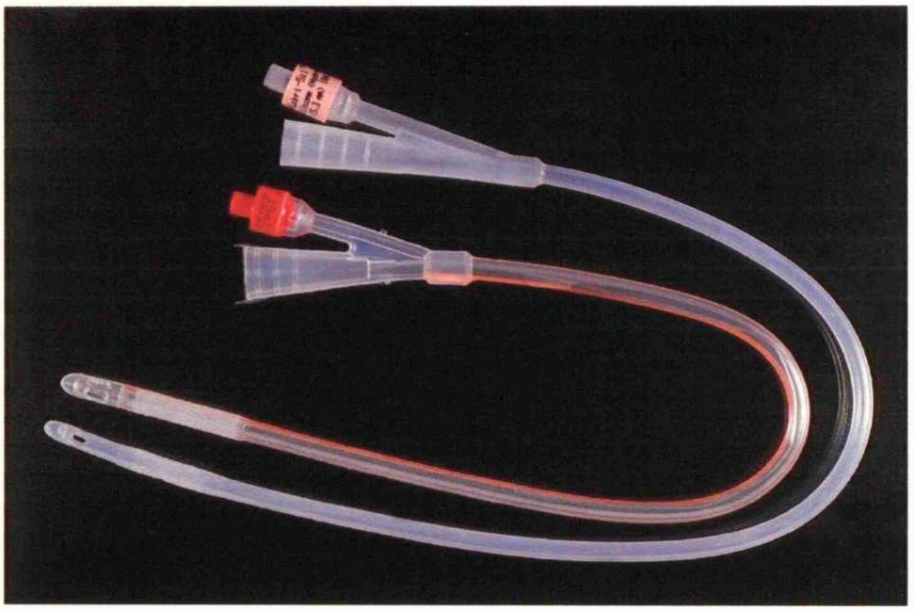


Diagram from Elving *et al.*, (2003)

Figure 1.6: Foley urinary catheters



- Measurement of urine production in intensive care patients
- Facilitates urethral repair following prostatic or gynaecological surgery
- Management of long-term urinary incontinence in elderly patients or those with spinal cord injuries or major neurological disease

1.3 Problems associated with biomedical devices

Biomaterials are designed to provoke a minimal immune response and regulatory criteria have been established to ensure the use of non-toxic materials free from haemolysins and other harmful substances (Bayston, 2003). Biomaterials are also designed to be durable because long-term use can lead to mechanical dysfunction which in some cases can be life threatening. Artificial heart valves, for example must be able to withstand repetitive stress as their failure would lead to heart malfunction. Artificial bone joints must also be highly durable and extensive research has been undertaken to identify strong, lightweight materials for this purpose. However, relatively little regard has previously been given to the role that biomaterials play in acting as a source of infection. Historically, biomaterials were not identified as a source of infection as the causative agents occurring on the materials were normally believed to be "harmless" bacteria found on the skin and mucous membranes. This meant their presence was often attributed to contamination of the material (Bayston, 2003).

At present, treatment of medical device-induced infection includes replacement of the device, which can be painful and time-consuming for the patient and also costly

to the health care provider. The alternative is the administration of antimicrobial agents. However, the effectiveness of such an approach can be variable, particularly with the increasing incidence of antimicrobial resistance and the reported enhanced resistance exhibited by microbes within biofilms (Gilbert and McBain, 2001; Costerton and Lashen, 1984).

1.3.1 Microbial colonisation of biomaterials

Biomaterials are at greater risk of microbial contamination than normal healthy tissues as they are non-ablative and therefore lack the ability to remove adherent microbes. In contrast, mucosa and skin constantly shed epithelial cells, and along with them, attached microbes. This ablative capability is effective in resisting microbial colonisation. Once a layer of microbes have become established on a biomaterial surface, in addition to cell multiplication of adherent cells, further microorganisms can attach resulting in accumulation. This gradual increase in the number of microorganisms eventually causes a structure to form known as a 'biofilm', comprising not only microbes but also extracellular polymeric substance (EPS; Section 1.6).

Despite silicone rubber exhibiting many of the properties necessary for a desirable biomaterial, it faces immense challenges in resisting biofilm formation. Silicone rubber is particularly hydrophobic (Everaert *et al.*, 1998a; 1998b), and research has shown that hydrophobic surfaces are less prone to microbial colonisation than hydrophilic surfaces (Everaert *et al.*, 1997; Busscher *et al.*, 1997). Despite this, biofilms are still capable of forming on silicone rubber.

1.4 Mechanisms of microbial adherence to biomaterials

The adherence of a microorganism to a surface is classically considered to be a two-stage process (Waters *et al.*, 1997), and involves a combination of specific and non-specific interactions. Non-specific interactions are believed to be reversible and the initiators of adherence. They include hydrophobic interactions and Van der Waals forces. The fact that these interactions are weak leads to the reversible nature of such adherence. The second stage of adherence involves specific interactions which are stronger and generally irreversible through gentle rinsing. Specific interactions are mediated through ligand-receptor bonds including protein-protein interactions.

1.4.1 Non-specific adherence mechanisms

Non-specific mechanisms of adherence permit the initial attraction between host surfaces and microbes thereby allowing specific and irreversible interactions to occur. Examples of non-specific adherence mechanisms are outlined below.

1.4.1.1 Electrostatic interactions

Electrostatic interactions (ion-ion interactions) and electrodynamic interactions include Van der Waals forces, London dispersion forces and hydrophobic interactions. All of these have been implicated in the initial stages of microbial adherence (Jones and O'Shea, 1994). Electrostatic interactions are thought to be largely repulsive whilst electrodynamic interactions promote adherence (Hobden *et*

al., 1995). If a negatively charged cell such as the yeast *Candida albicans* (Cotter and Kavanagh, 2000) approaches a negatively charged surface, binding will only occur if electrostatic repulsion is overcome by net attractive forces (Denyer *et al.,* 1993). These adherence interactions can be explained by the DVLO Theory. This theory was originally used to explain colloidal stability (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) and states that the total interactive energy of two smooth particles is determined solely by the sum of the Van der Waals attractive forces and the usually repulsive electrostatic forces. Particles in solution and surfaces submerged in aqueous suspension naturally acquire charge, which is exactly balanced by an equivalent number of counterions which leads to an electric double layer. The DVLO theory states that the net repulsive forces between two colloidal particles must be overcome by attractive forces in order for the particles to adhere. A review of the DVLO Theory is presented in Marsh and Martin (1999), which includes a diagram illustrating how the interactive forces between two particles alters with distance and highlights the reversible nature of these types of forces.

1.4.1.2 The hydrophobic effect

The Latin term 'hydrophobic' literally means 'a fear of water'. Most hydrophobic substances contain non-polar chemical groups which repel water in the same way that the hydrophobic heads of phospholipids repel water by forming a micellar structure.

Hydrophobic interactions are defined as 'reactions between two or more compounds in an aqueous phase, with the subsequent elimination of water molecules associated

with the interfacing molecules' (Jones *et al.,* 1991). Hydrophobic interactions are significant in the non-specific adherence of *Candida* to glass surfaces (Nikawa *et al.,* 1989) and hydrophobicity is also implicated in adherence of streptococci to glass (Satou *et al.,* 1988). Hydrophobic interactions are the basis behind the thermodynamic approach to adhesion (Klotz *et al.,* 1985; Minagi *et al.,* 1985). Microorganisms including viral particles have evolved ways to use the hydrophobic effect to adhere to substrata (Doyle, 2000). In fact, there is reason to believe that the hydrophobic effect is the most important driving force behind the adherence of pathogens (Duncan-Hewitt, 1990). Hydrophobicity in the context of materials can be described as the wettability of a surface; the less wettable, the more hydrophobic the surface. It is suggested that hydrophobic surfaces inhibit microbial adherence, (Everaert *et al.,* 1998a; Everaert *et al.,* 1998b). The surface free energy is related to the hydrophobicity with hydrophobic surfaces exhibiting low surface free energy.

1.4.2 Specific adherence mechanisms

Specific mechanisms of adherence involve ligand-receptor binding and are less likely to occur on 'naked' biomaterials (biomaterials devoid of a protein coat; Verran, 1999). However biomaterials may contain surface molecules which act as ligands for microbial cells. *In vivo,* biomaterials are unlikely to remain 'naked' for long since proteins from the surrounding interstitia rapidly adsorb to the substratum forming a protein conditioning film (Busscher *et al.,* 1997; Everaert *et al.,* 1998a; Taylor *et al.,* 1998). Many of the proteins within the film ultimately serve as receptors and ligands for microbial attachment (Verran, 1999). The protein portion of a yeast cell wall is important in the adherence process as revealed by Douglas

(1986), when *C. albicans* was pre-treated with a variety of proteolytic enzymes and reducing agents to alter the cell surface proteins. The result was an inhibition of *C. albicans* adherence (Douglas, 1986). The outermost layer of the yeast cell wall is comprised of mannoproteins and is thought to contain putative fungal adhesins and receptors (Pendrak and Klotz, 1995). *Candida albicans* has four known structurally related adhesins: Hwp1, Alalp/Als5p and Als1p which are often collectively referred to as glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWP; Sundstrom, 2002). Receptors are also found on the yeast cell wall and these bind to ligands on other cells and substrata leading to adhesion.

1.5 Factors influencing microbial adherence

Microbial and material surface properties all influence initial cell adherence. Factors affecting microbial adherence *in vivo* are shown in Table 1.2. Several of these are particularly relevant to the work in this thesis and are described in more detail below.

1.5.1 Biomaterial surface free energy

Material/substratum surface free energy relates to the hydrophobic effect and is known to influence microbial adherence (Section 1.4.1.2).

1.5.2 Surface topography

The topography of a biomaterial can have a significant influence on microbial adherence. The contribution of surface topography to attachment is believed to be

Table 1.2: Factors affecting microbial adherence

Factors influencing microbial adherence	**References**
Surface topography	Boyd and Verran, 2002
	Radford *et al.*, 1998
	Taylor *et al.*, 1998
	Yamauchi *et al.*, 1990
Presence of conditioning film, *e.g.*	
Saliva	Busscher *et al.*, 1997
Urine	Mobley, 1996
Hydrophobicity of material	Everaert *et al.*, 1997
Hydrophobicity of microorganism	Hazen, 1990
In vivo culture conditions of microorganisms	Critchley and Douglas, 1987
Strain of microorganism	Waters *et al.*, 1997

greater than physico-chemical interactions such as surface free energy and hydrophobicity (Quirynen *et al.,* 1990). The irregularities on a rough surface provide microbes with protection from shear forces. The increased surface area of a rough substratum may also lead to increased microbial attachment by providing more contact points (Holah and Thorpe, 1990; Leclercq-Perat and Lalande, 1994). It is very difficult to manufacture a completely smooth surface although manufacturing processes can be designed to reduce surface irregularities, *e.g.* casting silicone rubber against acrylic rather than dental stone results in a more uniformly smooth surface (Verran and Maryan 1991). Surface roughness can be quantified using a variety of parameters, however the most commonly used is 'Ra' (average roughness) which can be described in micrometer (μm) or nanometer (nm) units. The Ra value describes the arithmetic average departure of the profile from the centre line (Verran *et al.* 2003).

The *in vitro* adherence of microorganisms, including *C. albicans,* to denture base resins of varying surface topography was assessed by Yamauchi *et al.,* (1990). The three materials tested had Ra values of 1.12 μm, 0.22 μm and 0.09 μm, which were created using different polishing techniques. The adherence of *Candida* was lowest on the smoothest resin and highest on the resin with intermediate roughness. *In vitro* studies have been performed by Verran *et al.,* (1991, 1997) assessing the effect of surface topography on the adhesion of *C. albicans* and other microorganisms to acrylic and silicone. The findings were similar to those of Yamauchi *et al.,* (1990). Scanning electron microscopy (SEM) was used to show that cells were adhering mostly between surface ridges (Verran *et al.,* 1997) and it was later demonstrated that cells did not adhere in the large irregularities present on the roughest materials,

which suggested that this was because such irregularities were proportionally too large to offer protection to retained microbes (Verran *et al.*, 1991). Other studies (Radford *et al.*, 1998 and Taylor *et al.*, 1998) have demonstrated similar results.

1.5.3 Presence of conditioning films

Conditioning films can be defined as the non-microbial substance that adsorbs to surfaces from the surrounding environment. These conditioning films tend to be comprised of proteins, electrolytes and other non-microbial components. Conditioning films can have a large influence on the adherence of microorganisms through changing the surface energy and microrugosity of the substrata (Radford *et al.*, 1998). A conditioning film can also reduce the effect of the underlying material on microbial adherence (Taylor *et al.*, 1998). The protein components of the conditioning film can provide ligands or binding sites for microbes thus promoting biofilm formation. Conversely, conditioning films may also have a protective function and contain proteins such as histatins or antibodies that can inhibit the adherence of organisms (Tsai and Bobek, 1998). Conditioning films are present on surfaces in the oral cavity (Section 1.5.4) and on other mucosal surfaces. They also occur on ocular surfaces originating from conjunctival mucus and tears, which contain mucus, proteins, lipids, antibodies and lysozyme.

1.5.4 Salivary conditioning film

Saliva is a glandular secretion that constantly bathes oral surfaces. Saliva is produced by the three-paired major salivary glands (the parotid, submandibular and sublingual) the minor salivary glands and the gingival fluid (Gibson and Beeley, 1994).

1.5.4.1 Composition of saliva

Water constitutes 99.5% of saliva with protein largely accounting for the remaining component. Stimulated and unstimulated saliva contain different quantities of ions and proteins. Stimulated saliva originates from the parotid glands and contains almost five times the sodium content and twice the iodide content of unstimulated saliva. Stimulated saliva also contains a higher concentration of proline-rich proteins (PRPs; Ferguson, 1999). PRPs are known to promote the adherence of certain microorganisms to enamel and other oral surfaces (Gibbons *et al.*, 1991; Gibbons and Hay, 1989; Lamkin and Oppenheim, 1993). There are three classes of PRPs defined as acidic, basic and glycosylated (Bennick, 1982). Proline, glycine and glutamine account for between 70% and 88% of all the amino acids in PRPs and ensure the primary PRP structure is highly repetitive (Bennick, 1987). Hydrophobic amino acids are either absent or present in very small quantities. The acidic PRPs exhibit charge and structural polarity. The N-terminus is highly acidic, and includes two phosphoserine residues. The highly repetitive sequence starts at residue 53 and is dominated by proline, glutamine and glycine interspersed with a few basic residues (Wong and Bennick, 1980). Structural studies have shown that the basic PRPs are

similar in structure to the acidic PRPs although they may have a higher carbohydrate content (40%; Bennick, 1982). The glycosylated PRPs also have a carbohydrate content (Bennick, 1982), but glycosylated PRPs still have the characteristic high content of proline, glutamine and glycine.

1.5.4.2 Function of saliva and the salivary conditioning film

The function of saliva is primarily protective, through buffering effects, lubrication, humidification and remineralisation of teeth. The physical movement of saliva over an oral surface removes loosely attached microbial cells and debris (Gibson and Beeley, 1994). Immunological defence against bacteria, viruses and fungi are also mediated by saliva. The protective role of saliva against fungi is highlighted by the increased incidence of oral candidosis and dental caries in patients with reduced salivary flow (Chaushu *et al.*, 2000). Additional digestive functions are performed by saliva, for example enzymatic digestion, bolus formation and taste (Gibson and Beeley, 1994). The typical constituents of whole saliva are presented in Table 1.3.

A salivary conditioning film on biomaterials is known to have an influence on microbial adherence (Busscher *et al.*, 1997). It is still not clear whether the conditioning film increases or reduces microbial adherence. The influence of saliva on the adhesion of yeasts and bacteria was investigated by Busscher *et al.*, (1997) using a parallel plate flow chamber. The studies revealed that incubation of silicone rubber with saliva (1.5 h) reduced the attachment of yeasts and bacteria. The interactions formed were also weaker as shown by the dissociation of the microbes from the material after a passage of air through the chamber. The influence of saliva

on yeast adherence to silicone rubber was also investigated by Jones *et al.,* (2001) and a similar reduction in the adherence of *C. albicans* was observed. McCourtie *et al.*, (1986), pre-treated acrylic surfaces with saliva for 30 min and found reduced adherence of *C. albicans, C. tropicalis* and *C. glabrata.*

Conversely, other studies have shown an opposite effect with saliva. A significant increase in *C. albicans* adherence was reported after treatment of poly(methacrylate) with mixed human saliva (Edgerton *et al.,* 1993). This finding could be due to an increase in biomaterial surface microrugosity which was apparent on materials coated with the saliva. Nikawa *et al.*, (1992) found that an adhesive interaction between oligosaccharides on proteins and receptors/ligands on *C. albicans* appeared to mediate adherence to glass. Continued research performed by Nikawa *et al.,* (1997) further confirmed that salivary conditioning films enhanced fungal colonisation. PRPs have also been implicated in influencing microbial adherence. Research has demonstrated that *C. albicans* adheres to PRPs *in vitro* (O'Sullivan *et al.,* 1998). Also PRPs have been found to enhance the adhesion of certain strains of *Actinomyces viscosus* (Hay *et al.,* 1986) and *Streptococcus gordonii* to tooth surfaces (Gibons *et al.,* 1991). Current research has established that *in situ* medical devices readily become coated in conditioning films. The exact composition of these films is dependent on the location in the body. Some medical devices proceed to function normally without further problems. Others, particularly if the recipient is immunocompromised, demonstrate adherence of microorganisms. It is likely that accumulation of microorganisms will allow the development of a biofilm and at this stage, clinical infection or dysfunction of the device can occur.

Table 1.3: Typical constituents of whole saliva

Proteins	Features
Histatins	Antifungal and antibacterial properties Exhibit a candidacidal domain that has a potency comparable to that of synthetic antifungal agents
Proline rich proteins	Involved in pellicle formation and promote adherence of certain microbes
Albumin	Detected in very small amounts in orally healthy individuals Concentrations are significantly increased in-patients with gingivitis or periodontitis (Henskens *et al.*, 1993)
Statherins	Have a low molecular weight When adhered to hydroxyapatite, promote the adherence of a few oral bacteria such as *Actinomyces viscosus* (Gibbons and Hay, 1989)
Mucins	Provide the visco-elastic character to all the mucosal secretions (Van der Reijden *et al.*, 1993) Physiological functions include, cytoprotection, lubrication and protection against dehydration.
Cystatins	Endogenous proteinase inhibitors which protect tissues from proteolytic attack by microbes
α-Amylase	Abundant salivary component Main role is in digestion but also has growth inhibitory activity against *Neisseria gonorrhoeae* (Schenkels *et al*, 1995)
Secretory immunoglobulin A	Member of the adaptive immune system and is the predominant immunoglobulin of the secretory immune system
Lysozome	Lyses unwanted cells
Extra-parotid glycoprotein	Low molecular weight. Binds to several oral and non-oral bacteria (Schenkels *et al.*, 1993)

1.6 Biofilm formation

A biofilm is an organised community of microorganisms found colonising a surface. Biofilms occur on almost any surface supplied with adequate moisture and readily occur on aquatic substrata, water pipes and tanks and on the leaves of plants. In the case of water purification, the establishment of biofilms within the treatment system provides an essential function within the purification process by breaking down waste products. Biofilms arise through the initial adherence of a few pioneer colonisers to the substrata. Secondary colonisation occurs through the gradual recruitment of cells which co-adhere by various mechanisms (Section 1.4.2). Cells within a biofilm produce extracellular polymeric substance (EPS), which is often carbohydrate based and regarded as the major structural component of the biofilm matrix (Sutherland, 2000). Electron microscopy has shown that EPS forms an ordered array of fine fibres which provides the structural support of the biofilm. The EPS is gel-like and highly hydrated forming a thick coating around the microorganisms (Sutherland, 1997). The up-regulation of EPS synthesis occurs extremely quickly after attachment of a cell to a surface and is thought to be mediated through cell-cell signalling (Allison and Sutherland, 1987; Davies *et al.*, 1995). Biofilm development involves the multiplication of the attached microbes and the continual recruitment of planktonic cells from the surrounding environment. In the majority of natural habitats, biofilms comprise a variety of species and genera whereas in biomedical situations, monocultures are more common. However, this is not the case in areas such as the oral cavity, where many microbials species are present. Biofilms are comprised of up to 97% water (Zhang *et al.*, 1998) although this varies with the type of biofilm. The water component can be bound within the

capsules of microbial cells or can exist as a solvent where its viscosity is determined by the solutes dissolved within it (Sutherland, 2000). Other biofilm components include secreted polymers, absorbed nutrients and metabolites, products from cells lysis and even particulate material from the immediate surrounding environment. The typical composition of biofilms is shown in Table 1.4.

Table 1.4: Typical composition of a biofilm, adapted from (Sutherland, 2001).

Component	Percentage of biofilm
Water	Up to 97%
Microbial cells	2-5% (polymicrobial)
Polysaccharides, monosaccharides and heteropolysaccharides	1-2% (neutral and polyanionic)
Proteins (extracellular and resulting from lysis)	<1-2% (including enzymes)
Nucleic acid	<1-2% from lysed cells
Ions	bound and free

1.6.1 Biofilm structure

The precise structure of a mature biofilm varies depending on its location, the nature of the constituent organisms and the availability of nutrients (Stickler, 1999). Three biofilm variants have been suggested, based on their structure and are described as heterogeneous mosaic, dense confluent and penetrated water channel (Wimpenny and Colasanti, 1997). In general, higher levels of nutrients lead to much denser biofilms than those observed under oligotrophic conditions (Sutherland, 2001). Biofilm structure also varies with the hydrodynamic conditions (Cowon *et al.,* 2000). The shear rate will determine the extent of cell erosion and influence the physical morphology and dynamic behaviour (Cowon, 2000; Stoodley *et al.,* 1999).

1.6.2 Biofilms associated with specific medical devices

The oral cavity is a non-sterile environment and contains all the desirable features for microbial growth and colonisation; moisture, nutrients and colonising sites. The normal oral microflora is highly diverse and comprises of over 350 genera of culturable bacteria. Streptococcal species comprise 70% of the bacteria although this varies with the site investigated. In addition to bacteria, fungi of the genus *Candida* are frequent oral colonisers. The species *C. albicans* is perhaps the most common of these and it is estimated that up to 40% of people harbour *C. albicans* (O'Sullivan *et al.,* 1997). This organism is one of the main colonisers of denture-soft lining materials (Waters *et al.,* 1997) and voice box prostheses (Busscher *et al.,* 1997).

The urinary tract, in contrast to the oral cavity, is generally a sterile environment. However, patients undergoing long-term bladder catheterisation often experience urinary tract infections (UTIs). This can be caused by a number of bacteria and also yeasts. A common bacterium infecting catheters is *Proteus mirabilis* (Stickler 1999; Stickler *et al.*, 2002).

1.6.2.1 *Candida* and candidal biofilms

Candida albicans is a commensal of humans, primates and other warm blooded animals and is the principal opportunistic fungal pathogen of man (Odds, 1988). It is responsible for the disease candidosis, which most commonly occurs in the mouth and vagina and in its most frequent form is often referred to as 'thrush'. Yeast infections can also occur in the gut, bone joints and on the skin and nails (Odds, 1988). *Candida albicans* biofilm formation proceeds in an organised fashion and is similar in development to biofilm formation by many bacterial species (Chandra *et al.*, 2001). Formation of candidal biofilm is closely associated with the generation of EPS (Chandra *et al.*, 2001). There are four main types of oral candidosis (Table 1.5 and Figures 1.7 a-d). Oropharangeal candidoses are prevalent in patients with a compromised immune system, *e.g.* AIDS patients, bone marrow transplant recipients and patients undergoing cancer chemotherapy. However, oral candidoses are also frequently encountered in denture wearers due to the colonisation and retention of *C. albicans* to denture acrylic and denture soft lining materials.

Table 1.5: Four main types of oral candidosis

Type of oral candidosis	Symptoms
1. Acute pseudomembranous (Figure 1.7a)	Painful lesion usually on upper surface of tongue
2. Acute atrophic (erythmatous) (Figure 1.7b)	White plaque on mucosal surface
3. Chronic atrophic (erythematous) (Figure 1.7c)	Red lesions adjacent to fitting surface of upper denture. Can be asymptomatic
4. Chronic hyperplastic (Figure 1.7d)	White plaques on mucosal surface that can not be removed by scraping

Figure 1.7 a: Acute pseudomembranous candidosis

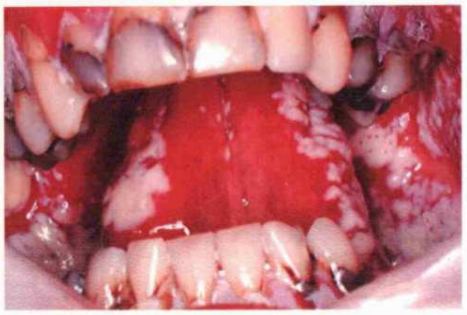


Figure 1.7 b: Acute atrophic (erythmatous) candidosis

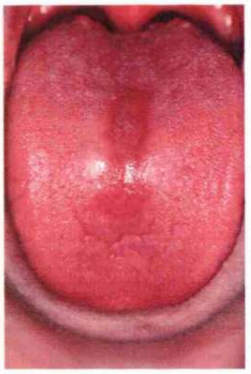


Figure 1.7 c: Chronic atrophic (erythmatous) candidosis

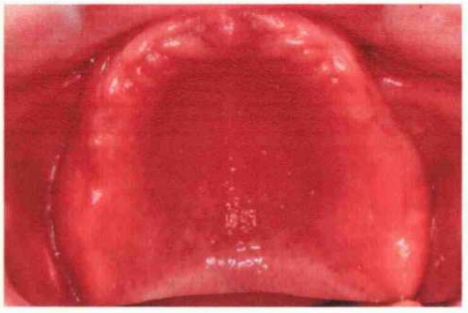
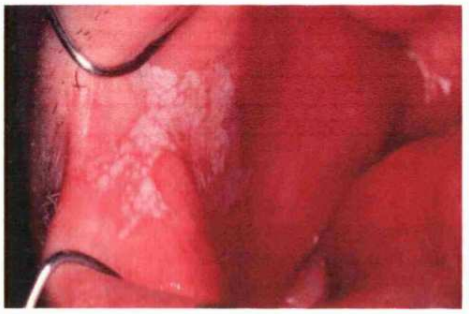


Figure 1.7 d: Chronic Hyperplastic candidosis



1.6.2.2 Biofilm formation on denture-soft liners

Biofilm formation on dentures tends to occur when there is a failure to adhere to regular hygiene regimens in relation to denture cleaning. Denture biofilm is also know as denture plaque. This problem is particularly evident amongst the elderly. Denture-soft liners are positioned in an area where stagnant pools of saliva can form and this provides an ideal reservoir for microorganisms. The most common microbe causing symptoms on soft liners is *C. albicans* and the formation of a candidal biofilm on these materials has been associated with the infection chronic atrophic candidosis. This infection presents as areas of erythema adjacent to the fitting surface of the denture. According to Ray (1987), chronic atrophic candidosis affects over 60% of denture wearers over the age of 65. As well as causing infection, *C. albicans* can also damage denture-soft liners by in-growth. Candidal in-growth is thought to be initiated by thigmotropism (contact sensing) to locate weak points within the material's structure and this is followed by hyphal penetration of the material (Busscher *et al.*, 1994; Eerenstein *et al.*, 1999).

1.6.2.3 Biofilm formation associated with voice box prostheses

Despite enabling restoration of speech, voice box prostheses have their drawbacks. As with the denture-soft liners, they are prone to colonisation by mostly *C. albicans* (Busscher *et al.*, 1997; Eerenstein *et al.*, 1999; Everaert *et al.*, 1998a; 1998b). The problem is accentuated in cancer patients because they are likely to be receiving chemotherapy or radiotherapy, which means they are immunosuppressed and this in turn, increases the presence of *Candida* in the oral cavity (Epstein *et al.*, 1993). As

with denture-soft liners, *Candida* causes deterioration of the prosthetic voice box by colonisation and invasion into the polymer. The hyphal forms of the organism are also responsible for blocking the valves of the device. The resulting blockage allows leakage of oesophageal contents into the trachea and increases the effort needed by the patient to produce phonation (Izdebski, 1987). Pulmonary infection may also occur due to leakage of fluids into the trachea. All of these problems lead to a reduced life-span for the device, which is typically only 3-4 months (Busscher *et al.*, 1997). Regular device replacement causes scarring and fibrosis of the tracheo-esophageal fistula which in turn causes shunt insufficiency and external leakage of fluids (Leunisse *et al.*, 1999).

1.6.3. *Proteus mirabilis* associated biofilms

Proteus mirabilis is a motile Gram-negative bacterium belonging to the family *Enterobacteriaceae*. The organism can undergo extreme morphological changes, from a short, single vegetative rod-shaped cell to an elongated, highly flagellated form referred to as a swarmer cell (Mobely, 1996). *Proteus mirabilis* is found in soil, polluted waters, and in the intestines of animals and humans. It has the ability to produce an urea-inducible urease that catalyses the production of ammonia and carbon dioxide from urea (Coker *et al.*, 2000). *Proteus mirabilis* is not a common cause of UTIs in healthy individuals, where more than 90% are caused by *Escherichia coli* (Rubin *et al.*, 1986). However, in patients with structural or functional abnormalities in their urinary tracts or in patients with long-term catheters, up to 44% of UTIs are caused by *P. mirabilis* (Warren, 1997). The bacteria are thought to enter the catheter via the drainage bag, which can easily become

contaminated. On contact with a solid surface, *P. mirabilis* differentiates into swarmer cells that collectively move across the catheter surface (Alavi and Belas, 2001). This behaviour is termed 'swarmer cell differentiation' and has also been demonstrated in other bacteria such as species of *Vibrio* (McCarter and Silverman, 1990) and *Serratia* (Alberti and Harshey, 1990). Swarming is a multicellular phenomenon used to seek new energy sources and to move away from a crowded centre. Swarmer cell differentiation is mediated through a physical sensing of a surface by an individual bacterium. It occurs via the inhibition of flagella rotation as the cell nears the surface.

1.6.3.1 Biofilm formation on urinary catheters

Biofilm formation on urinary catheters can occur within hours of insertion (Stickler, 1999). Colonisation is usually by bacteria from the periurethral skin and these often instigate UTIs. The bacteria migrate along the epithelial surface of the urethra and the external surface of the catheter entering the catheter via the taps of the urine drainage bag. The drainage bag eventually becomes heavily colonised and from these reservoirs, bacteria migrate to the drainage tube, the catheter and the bladder (Stickler, 1999).

In the first week of catheterisation, colonisation is usually by a single species such as *Proteus mirabilis, Staphylococcus epidermis, Enterococcus faecalis* or *Escherichia coli.* (Morris and Stickler, 1998). This initial colonisation is followed by the accumulation of additional species, mostly Gram-negative bacilli. The bacteria generate a biofilm on the external and luminal surface of the catheter, which when

observed by electron microscopy, demonstrate bacteria embedded in an extensive polymeric matrix (Ramsay *et al.*, 1989). Ohkawa *et al.*, (1990) found that 21 out of 28 catheters removed after 7-16 days were colonised with a biofilm. Further to this, a study carried out in the UK on 457 catheterised patients being cared for in the community, recorded 506 emergency referrals over a six month period, predominantly related to blocked catheters (Kohler-Ockmore and Feneley, 1996). Several surveys have reported that this complication is experienced by up to 50% of patients undergoing long-term catheterisation (Kunin *et al.*, 1987; Mobley and Warren, 1987). Catheter blockage occurs following colonisation with *P. mirabilis* or other urease producing species. Urease causes an elevated pH of the urine, due to the breakdown of urea, which promotes the crystallisation of calcium and magnesium ammonium phosphates. The crystals formed obstruct the catheter and cause trauma to the bladder and urethral mucosa upon withdrawal of the catheter. Blockage of the catheter lumen leads to urine retention and painful bladder distension. Urine retention can facilitate ascending infection of the urinary tract culminating in episodes of pyelonephiritis, septicaemia and shock. Undetected catheter blockage is particularly problematic for people in the community where professional care is not immediately available.

1.6.3.2 Implications of urinary catheter biofilm formation

Catheter-associated UTIs (CAUTIs) represent the single most frequently acquired nosocomial infection (Burke and Riley, 1996; Kunin, 1987; Stamm, 1991; Warren, 1997). On an annual basis, there are believed to be 100,000 cases of nosocomial infection in England, costing the UK National Health Service £1 billion each year

(Vincent, 2003). More serious problems occur if the infection remains untreated. Such problems could include pyleonephritis, septicaemia and even death. A study of hospitalised patients showed that the presence of a UTI (defined as > 10^5 organisms ml^{-1} urine) during bladder catheterisation was associated with a threefold increase in mortality (Platt *et al.*, 1982). Other complications associated with long-term catheterisation are presented in Table 1.6.

1.7 Biofilm control strategies

Physical methods can be used for the removal of biofilms from material surfaces and include mechanical scraping or cleaning, electrification, sonication and application of an ablative laser light (McBain *et al.*, 2000). These methods are all effective in biofilm removal, and are frequently used in an industrial setting for the treatment of water pipes and tanks. However, physical removal methods are not readily applicable to medical devices. Chemical control methods tend to be utilised more frequently in such instances, although these methods are notoriously ineffective. The limited effect of chemical biofilm control is, in part, due to the reduced effectiveness of antimicrobial agents on biofilms as opposed to planktonic phenotypes. McBain *et al.*, (2000), reported that biofilms exhibit a broad spectrum of resistance to antimicrobials and may be 1000-fold less susceptible than their free-living counterparts. There are a number of factors that contribute to the ineffectiveness of antimicrobial agents on biofilms. These include a reaction-diffusion limitation to the passage of antimicrobials across biofilms (Gilbert and McBain, 2001). This limitation arises due to the presence of EPS, which coats the cells and impedes the

Table 1.6: Complications associated with long-term urinary catheterisation

Complication	Details
Bacteriuria	Microbial colonisation of the bladder
	Can lead to uropsepsis and septicaemia
	Can occur within 2-4 h after catheter insertion
	Caused by the presence of a foreign object (catheter)
Urinary tract infection (UTI)	Most common complication
	Can occur at least twice a year
	More common in women then men
Urosepsis	Mortality due to urosepsis is 3 fold higher in catheterised patients (Kunin *et al.*, 1987)
	Catheter irrigation and use of prophylactic antibiotics have proven ineffective treatments
Kidney stones	Occur in 8% of patients with catheters (Newman, 1998)
Catheter obstruction	Occurs in 24-40% of patients with catheters; caused by bacteria collection, protein crystallisation or mucus plugs (Newman, 1998)
	Usually occurs around the tip of the catheter, the balloon or within the lumen
	Causes painful distension of the bladder

antimicrobial diffusion to the target cells. The presence of EPS and also extracellular enzymes can cause degradation of the antimicrobial before it reaches the cells. This is thought to occur with vancomycin and rifampicin, which although readily diffuse into the biofilm matrix appear to be inactivated by the time they reach the cells (Zheng and Stewart, 2002). Certain antibiotics including tobramycin do not penetrate biofilms and are believed to bind to EPS (Walters *et al.*, 2003). Deep-lying cells within biofilms are often severely nutrient-and oxygen-deficient. Carbon and nitrogen supplies become severely depleted and elements such as iron are removed by naturally occurring host compounds, such as transferrin and lactoferrin (Bayston, 2003). The lack of these nutritional components can lead to the expression of starvation phenotypes. Protein expression for non-essential processes such as cell wall synthesis are down-regulated in starvation phenotypes. Furthermore, the energy transport system is arrested and ATP synthesis is greatly reduced (McNamara and Proctor, 2000). These systems comprise many of the target sites for antibiotics and so may contribute to the resistance of microbes within biofilms. Starvation phenotypes are also suggested to have multi-drug efflux pumps which aid in expulsion of antimicrobials.

1.7.1 Current strategies for managing biofilm formation on oral medical devices

The most effective way of managing biofilm occurrence on denture-soft liners is appropriate oral hygiene. Mechanical cleaning of dentures and soft liners and regular soaking in a cleaning product such as Steradent (Main constituents: sodium bicarbonate, sodium carbonate and citric acid; Reckitt and Benckiser, Clevedon, UK) should help prevent microbial colonisation and clinical infection. However, the

majority of people who suffer from problems caused by denture colonisation are elderly patients who are unable to maintain regular cleaning regimens. A second management strategy is to replace the soft liners but this incurs extra cost to the healthcare provider and inconvenience to the denture-wearer.

Antimicrobial prophylactics have been used to prevent candidal colonisation of voice box prostheses. The administration of Amphotericin B lozenges four times daily has been shown to significantly prolong the functional lifetime of oral prostheses (Mahieu *et al.*, 1986). Prolonged use of antifungal agents however, is uneconomical and undesirable due to the possible induction of antifungal resistance (Vincent, 2003).

An alternative to antimicrobial prophylaxis, and potentially more acceptable, is the use of appropriate diets and probiotic foods. A current strategy undergoing investigation in Holland is the oral administration of probiotics, certain dairy products and caffeinated soft drinks. *In vitro* tests using an artificial throat model showed that buttermilk consumption three times daily for nine days reduced bacterial loads to 3% of the control amount and reduced yeast presence in the biofilm to 15% of the control. Probiotic yoghurt and semi-skimmed milk were shown to reduce bacterial growth but conversely resulted in enhanced yeast growth (Free *et al.*, 2000a). Caffeinated soft drinks have also been shown to reduce bacterial prevalence but again were shown to encourage yeast growth (Free *et al.*, 2000b). The bacterium *Lactococcus lactis* has been found to decrease yeast prevalence in the artificial throat (Free *et al.*, 2001; Van der Mei *et al.*, 1999), which could relate to the reported antimycotic properties of this particular bacterial species (Batish *et al.*, 1990).

However, an equivalent and concurrent reduction in bacterial growth was not evident with *L. lactis*. Although administration of specific diets may help reduce biofilm formation it does not solve the problem entirely. Other ways of trying to combat biofilm development have been to use antimicrobial agents specifically targeting either biofilm-specific phenotypes or the EPS (McBain *et al.*, 2000). These strategies have however encountered limited success and perhaps a better approach is to concentrate on ways to improve the medical device to try to stop microbes adhering initially. Much research has already been carried out to generate material surfaces that have a lower susceptibility to microbial adherence (Everaert *et al.*, 1998a; Waters *et al.*, 1997) and some have been used commercially (*e.g.* silver-coated urinary catheters).

1.7.2 Strategies for controlling biofilm formation on urinary catheters

One of the most important strategies for preventing biofilm formation on urinary catheters is appropriate management and regular replacement of the catheter. Guidelines are set outlining procedures designed to prevent biofilm occurrence (Newman, 1998). However, catheter management can breakdown in the environment of nursing homes or the domestic setting of incontinent patients reliant on visits from nurses. Such treatment failures often arise as a result of infrequent nursing visits, largely due to staff shortages.

1.8 Strategies aimed at reducing biofilm formation

1.8.1 Antimicrobial-impregnated surfaces

Antimicrobial impregnation of polymers results in a reservoir of antimicrobial agents which are gradually released into the surrounding environment. An example of such a strategy is the use of the antifungal agent clotrimazole, which has been incorporated into silicone rubber facial prostheses (Pigno *et al.*, 1994). The purpose of this approach was to minimise the growth of a *Penicillium* fungus around the nasal areas of the prostheses and *in vitro* tests proved successful (Pigno *et al.*, 1994). A potential problem with this method is that the impregnated antimicrobial often leaches from the polymer too quickly and as a result this system does not have a long-term effect on biofilm formation. Chlorhexidine gluconate has been incorporated into denture base materials with the aim of providing a constant antimicrobial presence in the locality of the denture (McCourtie *et al.*, 1985). However, in these studies, rapid leaching of chlorhexidine from the polymer was again evident (McCourtie *et al.*, 1985). The release mechanism for chlorhexidine was diffusion and this may have caused the leaching. Other release approaches include, the 'polymer erosion mechanism' where a biodegradable polymer is used which releases the antimicrobial upon degradation (Lin *et al.*, 2001). However, in certain circumstances the accumulation of polymer debris from degradation can trigger inflammation and other associated problems (Lin *et al.*, 2001). In the oral environment, such a situation is of lower concern as silicone rubber debris would be removed through consumption. Environmental factors can also be exploited for drug release such as changes in pH or hydrophobicity acting as triggers for the release

mechanism. Studies have been performed using urinary catheters impregnated with the antiseptic, nitrofurazone (Maki *et al.*, 1997) and the broad-spectrum antibiotics minocycline and rifampicin (Darouiche *et al.*, 1999). All of the catheters exhibited significant reduction in catheter-associated UTIs, although the studies were small and resistant bacterial strains were not included in the tests. Limiting factors for impregnated biomaterials are fluid flow which often washes away the antimicrobial from its site of action, interaction with factors in the perfusion fluid, failure of release agents impregnated into the polymer and secondary factors such as the inability to sterilise the processed polymer (Bayston, 2003). A further factor causing failure is development of microbial resistance, which is an argument often used against the use of impregnated surfaces, particularly in situations where sub-lethal doses are administered via the material (Bayston, 2003). Another important factor to be considered is changes to the bulk physical properties of the biomaterial once it has been impregnated with an antimicrobial agent. Biomaterials are chosen because of their tough and durable nature, but the incorporation of additional chemical agents can weaken their structure, possibly reducing tear strength and durability. Furthermore, swelling could be promoted due to enhanced water absorption. These concerns often limit the amount of agent that can be incorporated and the type of biomaterial that can be used. For example, it would be very difficult to incorporate an agent into titanium whilst it would be relatively simple in a polymer such as silicone rubber.

1.8.1.1 Organo-silver complexes and silver ions

Silver complexes tend to have very high antimicrobial activities and possess very low water solubilities. However, the value of these complexes *in vivo* is often impaired due to the rapid development of protein conditioning films, which mask coated surfaces (Bayston, 2003). This occurs due to the extremely high affinity of proteins for silver ions (Bayston, 2003). In addition, *in vivo,* silver ions are very quickly converted to silver chloride through the bonding of chloride ions present in plasma; this form of silver is inactive and insoluble (Bayston, 2003). Commercially available medical devices that are coated with silver complexes have been developed, although their performance has been disappointing. Clinical trials have been performed to test the effectiveness of a silver oxide-coated urinary tract device (Riley *et al.,* 1995). The results showed not only a failure in preventing UTIs, but an actual increase in the incidence of catheter-associated infections for male participants. In a study of vascular catheters impregnated with silver sulphadiazine and chlorhexidine (Schmitt *et al.*, 1995) complete loss of antibacterial activity was evident after ten days. It has therefore been concluded by several researchers that silver impregnated biomaterials cannot currently be justified for clinical use (Bayston, 2003; Schierholz *et al.*, 1998).

1.8.2. Biomaterial surface modification

An obvious advantage of surface-modification techniques is that they are unlikely to affect the bulk properties of the biomaterial. Some surface modification techniques are aimed at preventing the initial adherence of microorganisms, whilst others are

designed to be antimicrobial. Surface modification techniques attempt to alter the characteristics of a surface known to be involved in adhesion. For example, surface hydrophobicity significantly influences microbial adherence (Everaert *et al.*, 1997; Everaert *et al.*, 1998b; Lassen *et al.*, 1994; Miyaki *et al.*, 1986).

1.8.2.1 Modification of surface chemistry

Poly(ethylene oxide) (PEO) applied to a surface is known to reduce protein adsorption (Desai and Hubbell, 1991; Johnston *et al.*, 1997; Prime and Whitesides, 1993), and biomaterials treated in this manner have been shown to inhibit the adherence of bacteria *in vitro* (Desai *et al.*, 1992; Humphries *et al.*, 1987; Johnston *et al.*, 1997). Gottenbos *et al.*, (2002) covalently coupled quaternary ammonium groups to silicone rubber. Quaternary ammonium groups have disinfectant properties (Hugo and Russel, 1992), and since these groups were covalently attached to the surface, long-term effects were envisaged. The surface modification was designed to be bacteriocidal and live/dead fluorescent stains were used *in vitro* to assess the effectiveness of these groupings on the material surface. The material was also tested *in vivo* by inserting discs into subcutaneous pockets on the backs of rats following pre-incubation with *Staphylococcus aureas.* Untreated silicone rubber, pre-incubated with the bacterium was used as a control. The results showed that seven out of eight controls resulted in infection, with only one out of eight of the surface-treated discs being infected (Gottenbos *et al.*, 2002).

Surface chemistry can inhibit microbial adherence through several mechanisms. Changes in hydrophobicity have already been discussed, however, chemical

groupings could conceivably mask attachment sites, or alter surface charge. In addition, the incorporation of chemical groups to a material surface could produce a more mobile chemical environment, particularly if long chains of functional groups are introduced i.e. long functional groups have more molecules increasing the occurrence of bond movements which will make the molecule more mobile.

Modification of surface chemistry using silanes

Silanes are substances which can be incorporated onto a material in order to alter the surface functional groups. Generally, inert materials such as silicone rubber need to be pre-treated before silane treatment in order to activate the surface. This is usually performed using argon plasma discharge treatment, although other forms of plasma treatment can also be used. Silver *et al.*, (1999) reported on the production of a haemocompatible biomaterial. To achieve this, silicone rubber was treated using oxygen plasma discharge treatment. The activated surface was then exposed to alkyltrichlorosilanes with different terminal functionalities. Static contact angle measurements and X-ray photoelectron spectroscopy (XPS) were used to verify the presence of the functional groups on the surface. Previous researchers have also attempted to alter the hydrophobicity of material surfaces using silanes in order to prevent microbial adherence to medical devices (Everaert *et al.*, 1998b). Research has also been carried out to investigate the effects on microbial adherence following plasma treating silicone rubber alone, with no subsequent silane treatment. Everaert *et al.*, (1998a), produced a silicone rubber voice box prostheses that was half treated by argon plasma discharge to generate a hydrophilic surface. The untreated silicone portion retained its hydrophobic characteristics. Test prostheses treated in this

manner were inserted into patients and after four weeks were removed and the presence of a biofilm determined. The results revealed that the original hydrophobic section of the prostheses had significantly less biofilm formation compared to the hydrophilic area of the prostheses. The surface of the silicone rubber was then treated to render it more hydrophobic (Everaert *et al.*, 1998b). This was performed again using argon plasma discharge to oxidise the surface, but was followed by the incorporation of fluoro-alkylsiloxane groups through silane immersion. Two fluorinated alkyltrichlorosilanes (CF_3 and C_8F_{17}) were chemisorbed to the surface and increased the surface contact angle (hydrophobicity) from 115° to 125° for CF_3 and to 140° for C_8F_{17}. The material surfaces were then tested for microbial adherence after being placed in a parallel plate flow chamber for four hours with *Strep. salivarius, Strep. epidermis, C. albicans* and *C. tropicalis*. In these experiments, the hydrophobic surfaces had slightly less bacterial and yeast adherence when compared with untreated silicone rubber. A more interesting finding however, was that the percentage detachment of microbes after the passage of an air bubble through the flow cell was a lot higher on the surface-treated material indicating a weaker attachment of the biofilm to the material. It is not fully understood why increasingly hydrophobic surfaces display less biofilm formation. It could relate to a direct influence on the microorganisms, or may act indirectly by reducing the presence of protein conditioning films. A theory by van der Mei *et al.*, (1993), stated that the composition, structure and cohesive strength of salivary conditioning films in the oropharyngeal cavity is dependent on the hydrophobicity of the underlying substratum. This suggests that conditioning films on hydrophobic surfaces have relatively low cohesive strengths thus permitting biofilm detachment. The presence of a conditioning film reduces the effects of the physico-chemical properties of the

underlying surface (Bayston, 2003; Schneider and Marshall, 1994; Taylor *et al.*, 1998; van Dijk *et al.*, 1988), and because conditioning films are the initial coatings that develop on a material, it is possible that the relative hydrophobicity of the surface acts indirectly on microbial adherence by affecting the composition or adhesive strength of the conditioning film. It may also be possible however, that the same surface properties preventing the microbes adhering to the surface may also prevent protein adsorption (Johnston *et al.*, 1997).

1.8.3 Topographical modification of surfaces

Modification of surface topography may also be utilised to minimise initial cell retention. The effect of surface roughness is variable and dependent upon the size of the defects causing roughness. It is generally acknowledged that an increase in surface roughness increases microbial retention to a certain point, and excessive roughness where surface defects are much larger than the attaching microbe cause reduced attachment (Verran *et al.*, 2003). Maximal attachment occurs in cases where the surface defects are the same size or slightly larger than the microbe (Verran *et al.*, 2003). It follows that below a minimum roughness microbial cells will not be retained, although this minimum roughness will obviously be dependent upon the size of the microbe (Bollen *et al.*, 1997; Verran and Boyd, 2001). These effects are only observed in the early stages of microbial colonisation. During the later stages, there is little difference between the amount of biofilm on both smooth and rough surfaces (Verran, 2002). By manufacturing surfaces so that they are compliant with a specified level of roughness, it may be possible to minimise biofilm formation generated by initial physical (as opposed to chemical) retention.

1.8.4 Alternative modification strategies

In 1984, Hayward and Chapman suggested that biomaterials coated with lipids similar to those located on the external surface of erythrocytes would be haemocompatible and should therefore be incorporated on to medical devices. This type of approach has been termed biomemesis (Williams, 1995) *i.e.* abstracting design from nature. The study of Haywood and Chapman (1984) used medical devices coated in phosphoryl-choline (PC), which as a result were extremely hydrophilic. As a consequence, it was proposed that water molecules would bind tightly to the PC head groups making it difficult for proteins and other materials to interact with the surface. It was further suggested that repulsion of a conditioning film may prevent microbial colonisation. This theory was tested by Stickler *et al.*, (2002) using PC co-polymerised with hydrophilic long-chain alkyl methacrylates, which were incorporated on to urinary catheter surfaces. The catheters were tested in a bladder model for *P. mirabilis* induced colonisation and encrustation. The PC-coated catheters were compared to that of other commercially available catheters which were assessed simultaneously. The results displayed no significant improvement in the blockage time or the extent of encrustation with the PC-coated catheters.

Some studies have attempted to produce surfaces that encourage protein adsorption. For instance, albumin is documented to be a "comfort surface" for phagocytes that perform bactericidal functions (Lin *et al.*, 2001). To encourage the adsorption of albumin on to a surface, high molecular weight dextran:cibacron blue was

incorporated within the polymer and also bonded to the surface. The dextran was designed to bind native albumin from blood and tissue fluid, thereby allowing the medical device surface to be selective for albumin *in vivo* (Lin *et al.*, 2001). Published results suggested this surface coating was less adherent to *Staphylococcus epidermis* (Keogh and Eaton, 1994). However, for immunocompromised patients whose defensive cells show abnormal bactericidal activities, the efficacy of this albumin affinity remains to be proved (Lin *et al.*, 2001).

1.9 Aims

The specific aim of this research was to develop a novel silicone rubber surface that was inhibitory to the adherence of both *Candida albicans* and *Proteus mirabilis*. The chosen methodology was to use argon plasma discharge treatment supplemented by chemisorption of specific chemical functional groups. All surfaces were characterised using contact angle determination, atomic force microscopy, and X-ray photoelectron spectroscopy and the chosen microbial adherence assays included both static and flow-through systems.

Chapter 2

Silicone rubber production, surface modification and characterisation

2.1 Introduction

Medical devices play a vital role in modern medicine. Due to their close proximity to biological systems, the material from which medical devices are constructed is of great importance. Biomaterials must be biocompatible, non-toxic and durable, and also require other properties depending on their function (Chapter 1, Section 1.1). Silicone rubber is a typical biomaterial used for medical devices and possesses excellent physical properties. In fact, of the many synthetic materials available, silicone rubbers are among the few that are adequately biocompatible and biodurable for use in implants and other biological applications. Detail on the structure and chemistry of silicone rubber is given in Chapter 1, Section 1.2.

Despite the benefits of silicone rubber as a biomaterial, there are associated problems related to its use. These problems are not just limited to silicone rubber but occur with all biomaterials including latex, poly(vinylchloride), polyurethane and acrylic. As discussed in Chapter 1, Section 1.6, the main problem occurs when silicone rubber becomes colonised with microorganisms, causing either clinical infection or destruction of the medical device. Previous research has attempted to combat this by developing a silicone rubber that is less susceptible to microbial colonisation. Frequently, this approach has involved the incorporation of antimicrobial agents into the silicone polymer. This may, however cause alterations to the bulk properties of the material, or as recent research has found, promote microbial resistance (Chapter 1, Section 1.8). An

alternative method of preventing microbial colonisation is to alter only the surface of the silicone rubber, leaving the bulk properties of the material unaltered.

It is widely acknowledged that alteration of a biomaterial's surface characteristics, can influence the ability of a microorganism to adhere to a surface (Busscher *et al.*, 1997; Everaert *et al.*, 1998a; 1998b). Previous research has demonstrated a variety of influential surface characteristics, which are discussed in detail in Chapter 1, Section 1.5. The methods available for manipulating these surface properties are discussed in Chapter 1, Section 1.8 and briefly outlined in Table 2.1.

2.1.1 Plasma discharge-silane treatment process

Silicone rubber is a particularly inert medium due to the presence of stable methyl groups on its surface. It is therefore difficult to initiate chemical reactions without firstly creating a chemically reactive surface. This can be achieved by plasma discharge treatment (Everaert *et al.*, 1998a; Ferguson *et al.*, 1993; Ratner, 1997). Plasma discharge treatments were first used on biomaterials in the 1960s and are particularly useful for enhancing biostability, improving lubricity and altering material wettability (hydrophobicity). The treatment involves the bombardment of a surface with ions, which remove surface functional groups, leaving a charged and hence reactive surface.

In this project, silicone rubber was exposed to argon plasma discharge treatment in a vacuum. The argon ions physically remove methyl groups from the silicone rubber

Table 2.1: Surface characteristics that can be altered and methods of alteration.

Surface characteristic that can be altered	Technique for alteration
Topography	Polishing (Yamauchi *et al.*, 1990; Verran *et al.*, 1997; Verran *et al.*, 1991).
Surface chemistry	Silane treatment (Everaert *et al.*, 1998b). Addition of quaternary ammonium compounds (Gottenbos *et al.*, 2002). Addition of polyethylene oxide (Johnston *et al.*, 1997).
Hydrophobicity	Plasma treatment (Everaert *et al.*, 1998a). Silane treatment (Everaert *et al.*, 1998b).

surface, and on re-exposure to air, oxygen atoms bond to the surface replacing the methyl groups. These oxygen atoms react readily when exposed to silanes. Silane molecules of the general formula $Cl_3Si(CH_2)_2R$ (R= specific functional group to be incorporated in order to change the surface properties) contain a silicon atom, a leaving group(s) (usually chlorine) and a functional group which is the desired chemical group present on the surface after treatment. On exposure to the plasma-treated silicone rubber, the silicon atoms on the silanes bond to the reactive oxygen groups forming siloxane bonds, whilst the chlorine atom(s) simultaneously leave(s) the silane. Siloxane bonds have a high degree of flexibility compared to the carboxyl and carbon-carbon bonds found in organic polymers and the functional groups therefore have a certain level of mobility. The functional groups are attached to the rubber by a siloxane bond which is linked to an ethyl group (rubber-O-Si-CH_2-CH_2-R). The ethyl group separates the functional group(s) from the silicone surface, thus providing stability that would otherwise be lacking due to the presence of adjacent electronegative atoms such as fluorine and the silicone rubber. The functional groups chemisorbed to the silicone rubber determine the characteristics of the surface. For instance, a surface with functional groups containing electronegative atoms such as fluorine or chlorine will be hydrophobic (Chaudhury, 1993; Everaert *et al.,* 1998b; Silver *et al.,* 1999), whereas functional groups such as ethylene oxide ($-OCH_2CH_2)_nOH$) or carboxylate (-COOH) will be more hydrophilic (Silver *et al.,* 1999). The surface mobility will be altered following the addition of functional groups as they are attached to the flexible siloxane bond. Functional group chain length is also likely to affect surface mobility, as is the density of the surface treatment. Previously, researchers have described this type of

surface treatment as the construction of 'self assembled monolayers' (SAMs) (Chaudhury, 1993; Ferguson *et al.,* 1993; Silver *et al.,* 1995; 1999). Furthermore, Everaert *et al.,* (1998b) stated that trichlorosilane molecules not only react with argon plasma-treated silicone rubber, but also have the ability to polymerise, yielding relatively thick siloxane layers. Everaert *et al.,* (1998b), hypothesised that the chemisorption of fluoro-trichlorosilanes to the rubber produced thick dendritic wedge structures, which occur particularly with longer chain fluoro-siloxanes. The proposition arose from X-ray photoelectron spectroscopy data which demonstrated layers greater than a monolayer thick (5-10 nm; Everaert *et al.,* 1998b).

2.1.2 Surface characterisation techniques

2.1.2.1 Contact angle determination

Contact angle measurements are often used to assess surface hydrophobicity. When a surface is wettable (hydrophilic) the contact angle is small, for example, the water contact angle of glass is 0° indicating its high wettability, whereas the average water contact angle of silicone rubber is 115 ± 3° (Everaert *et al.,* 1998b) highlighting that it is not wettable *i.e.* it is hydrophobic. There are several different methods for contact angle analysis, including the sessile drop technique, where a needle is placed in a water droplet (1-1.5 μl) on the material to be tested. The needle is then carefully moved until the contact angle appears to be maximal. The contact angle is then measured using a contour monitor. An alternative method is the static contact angle technique, where a

small drop of water is placed onto the test material, which is subsequently photographed or analysed by a video microscopy system to enlarge the image. Tangent lines are then drawn at the intersection of the drop and the material surface by geometric construction directly on the photograph or on a replica of the video image to estimate the contact angles. A dynamic contact angle method is also available, where a buoyancy graph is produced of a material as it is immersed in the wetting medium at a standard speed. The buoyancy graph represents the solid/liquid/air interface interactions as a function of time and immersion depth. Advancing contact angles are measured as the sample moves into the wetting medium and receding angles as the sample is withdrawn. Various polar and non-polar liquids are available for the measurement of contact angles. For example, water is a commonly used polar liquid and bromonapthylene, a non-polar liquid (Cahn Application Software manual, Cerritos, USA, 1980).

2.1.2.2 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy is a technique designed to determine surface chemistry and was employed in this study. Several researchers have utilised this technique to monitor surface modifications (Durand *et al.*, 2002; Everaert *et al.*, 1998b; Ferguson *et al.*, 1993; Ma and Chung, 2001; Silver *et al.*, 1999). XPS utilises X-rays, which have enough energy to eject core-level electrons from an atom. Since the energy it takes to remove a core electron is different for each atom, chemical identification can be achieved. XPS only detects the first few nanometers of a sample surface, which makes it an ideal technique for monitoring surface modifications. It can determine the

stoichiometry of a surface which makes the technique quantitative, but is unable to directly determine surface structure.

2.1.2.3 Analysis of surface roughness using atomic force microscopy (AFM)

Atomic force microscopy (AFM) is an imaging technique that allows high-resolution, three dimensional imaging and was utilised in this study to assess surface topography. The technique can generate molecular, and in some cases, sub-molecular resolution. The atomic force microscope consists of a probe tip connected to a cantilever. The probe is typically 10 nm in diameter and is passed across the surface of a sample to detect changes in height which are recorded by a laser beam reflected off the cantilever. A three dimensional image of the surface can then be obtained. There are several operating modes that can be used including a tapping mode and contact mode. The present study utilised the tapping mode, which involves vertical oscillation of the cantilever at a high frequency. Interaction of the tip with the sample causes attenuation of the oscillation amplitude, which is used to monitor height changes. AFM is an ideal method for identifying biomaterial surface topography as, unlike other surface visualisation techniques such as scanning electron microscopy (SEM), it also calculates surface roughness. The Ra value is the most commonly used parameter for surface roughness (Verran and Boyd, 2001) and describes the arithmetic average departure of the profile from the centre line. The Z-range (Rz) is the average distance in height between the five lowest and five highest areas on the section being examined. Both parameters are measured either in nm or μm, depending on the extent of surface

roughness. AFM is a highly sensitive technique and, as mentioned, can provide Ra values on a nanometer scale which is more difficult for other techniques such as solid stylus profilometry. However, AFM can only scan very small areas (up to 100 μm^2) thus irregularities in the topography of a surface can skew results unless a large number of measurements are made (Verran and Boyd, 2001).

Many researchers have used AFM to visualise and gain specific information about biomaterials including surface topograpghy and other surface features (Bai *et al.*, 1997; Bowen *et al.*, 2001; Boyd and Verran, 2002; Siedlecki and Marchant, 1998; Verran *et al.*, 2003).

2.1.2.4 Assessment of water absorption

The absorption of water into a polymer is an important factor in relation to its performance, as water uptake can induce swelling, which can distort the shape of the device leading to dysfunction. The extent of water absorption in to silicone rubber is dependent on several factors including its porosity and the type of filler used, which generally influences the overall hydrophobicity of the polymer (Waters *et al.*, 1996). It is also possible that surface characteristics may influence the uptake of water.

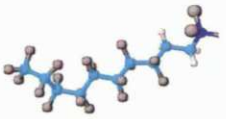
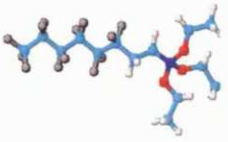
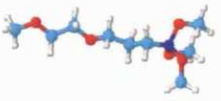
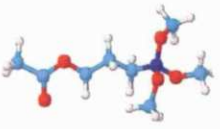
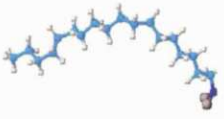
2.1.2.5 Bonding of silicone rubber to acrylic

In certain medical devices, silicone rubber needs to be firmly adhered to acrylic (e.g. denture-soft liners). Peel tests determine the force required to peel apart two adhered materials using a tensile testing machine, which pulls the materials apart at a constant rate until separation.

2.1.3 Choice of silanes for surface treatment

For surface treatment, eight different commercially available silanes were selected to treat the silicone rubber. Six initial treatments were tested (conveniently named T1 – T6) and then two further treatments (T7 and T8) were used to help determine the mechanisms of microbial adherence to the treated surfaces (Chapter 3, Section 3.5.1). The initial six silanes were chosen for their varying hydrophobicity and the aim was to produce a sequence of surfaces ranging from hydrophilic to hydrophobic, as past research had demonstrated a correlation between microbial adherence and material surface hydrophobicity (Chapter 1, Section 1.4.1.2). Each silane consisted of a standard structure which, contained a leaving group(s), a silicon atom and ethyl groups, which were connected to the functional group to be bonded to the surface. The chemical structures and formulae of the silanes used are shown in Table 2.2 and their structures upon incorporation onto the silicone rubber surface are shown in Figure 2.1. T1 silane was 1H,1H,2H,2H-perfluorodecyltrichlorosilane, which had Cl_3 as the leaving group and the functional group contained 17 fluorine atoms bonded to carbon atoms in a chain

Table 2.2: Silanes used for silicone rubber surface treatments including formulae and structures.

Silane name	Formula	Structure
T1: 1H,1H,2H,2H-perfluorodecyltrichlorosilane (hydrophobic functional group)	$CF_3(CF_2)_7CH_2CH_2SiCl_3$	
T2: (Tridecafluoro-1,1,2,2-tetrahydrooctyl)triethoxysilane (hydrophobic)	$CF_3(CF_2)_5CH_2CH_2Si(OC_2H_5)_3$	
T3: 2-methoxy(polyethyleneoxy)propyltrimethoxysilane (hydrophilic)	$CH_3O\text{-}(CH_2CH_20)_6\text{-}(CH_2)_3Si(OCH_3)_3$	
T4: Acetoxypropyltrimethoxysilane (hydrophilic)	$CH_3COOCH_2(CH_2)_2Si(OCH_3)_3$	
T5: N-octadecyltrichlorosilane (hydrophobic)	$CH_3(CH_2)_{16}CH_2\text{-}SiCl_3$	
T6: (333-trifluoropropyl)trichlorosilane (hydrophobic)	$CF_3CH_2CH_2SiCl_3$	
T7: Hexyltrichlorosilane (hydrophobic)	$CH_3(CH_2)_4CH_2SiCl_3$	
T8: Methyltrichlorosilane (hydrophobic)	CH_3SiCl_3	

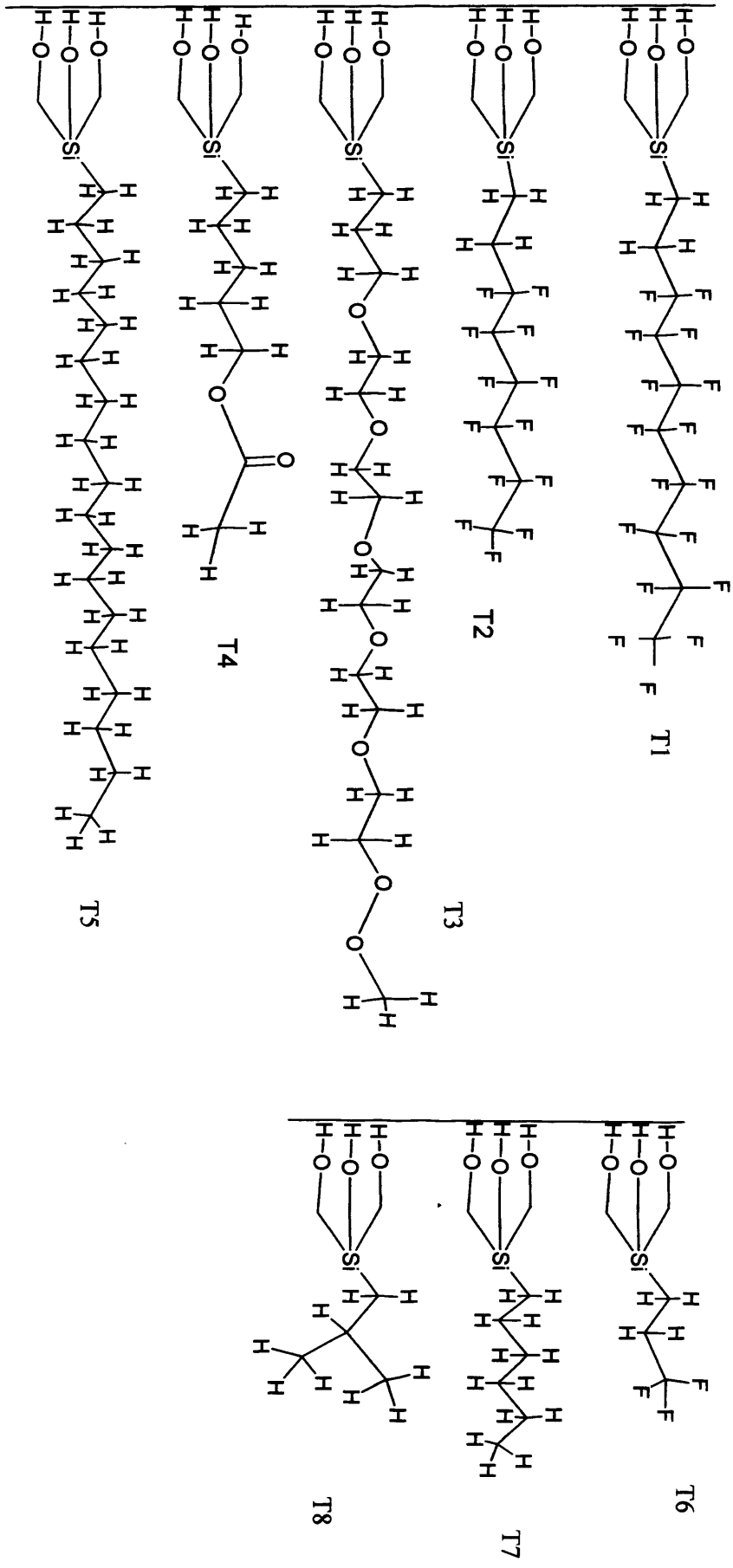


Figure 2.1: Schematic diagram of silanes chemisorbed on silicone rubber. Functional groups are attached via siloxane bonds.

structure. T1 was the most hydrophobic silane used due to its high fluorine content. The T2 silane was (tridecafluoro-1,1,2,2,tetrahydrooctyl)triethoxysilane. This was also hydrophobic due to a chain of 13 fluorine atoms attached to carbon. This silane had three ethoxy leaving groups. T2 silane contained less fluorine than T1 and had a shorter functional group. The aim of modifying a surface with T2 silane was to produce a slightly less hydrophobic surface than T1 and also to assess the influence of chain length. The T3 silane was 2-methoxy(polyethyleneoxy)propyltrimethoxysilane and contained 3 methoxy molecules as its leaving group and a poly(ethylene oxide) (PEO) functional group which in total consisted of 6 ethoxy groups and a terminal methoxy group. The functional group contained a total of 7 oxygen atoms. This silane was selected in order to produce a hydrophilic surface, which could be used as a comparison against the hydrophobic treatments. It is also recognised as an inhibitor of protein adsorption and as a result has been incorporated onto surfaces by a number of researchers (Chapter 1, Section 1.8.2.1). T4 silane, which was acetoxypropyltrimethoxysilane was also selected to produce a hydrophilic surface. It had the same leaving group as T3 but contained a functional group with only 2 oxygen atoms. Silane T5 was N-octadecyltrichlorosilane, which had Cl_3 as a leaving group and the functional group consisted of an 18-carbon long hydrocarbon chain making it the longest functional group chain of all the silanes used. The T5 surface treatment did not introduce chemically different atoms on to the surface but the treatment produced a physical and structural surface alteration rather than a chemical one. Octadecyl structures have been previously shown to inhibit protein adsorption onto surfaces (Arkles, 1980). T6 silane contained a very short functional group consisting of only 3

fluorine atoms attached to 1 carbon and the leaving group was Cl_3. The T6 silane was used to produce a slightly more hydrophobic surface than standard silicone rubber. As well as producing chemical changes on the surface of silicone rubber, the treatments also provided structural alterations and changes in surface mobility. For example T5 treated material contained a functional group that was 18 carbons long, which was a large structural change to the surface compared to the single methyl groups that naturally occur on the surface of silicone rubber. T6 material had a much shorter chain with only 3 fluorine atoms attached to one carbon which would be similar in structure to that of a methyl group. These variations in chain length provided a further variable that could have possibly influenced microbial adherence. The increase in surface mobility due to the addition of a siloxane bond was a further variable that may have influenced adherence with longer chains hypothesised to produce more mobile surfaces than those with shorter chain functional groups. Silanes T7 and T8 were chosen for the purpose of assessing whether chain length was a factor in influencing adherence or whether it was purely the presence of a different functional group. Similar to T5, the T7 and T8 silanes contained no chemically different atoms to those already found naturally on the silicone rubber surface. T7, which was hexyltrichlorosilane contained a chain of 5 alkyl groups ending in a methyl group and T8, which was methyltrichlorosilane was a much shorter version of this with only one methyl group. T8 was designed to imitate as closely as possible the silicone rubber surface.

2.2 Objectives

- To use argon plasma discharge treatment and silane chemisorption in order to produce silicone rubber materials with a range of chemically altered surfaces.
- Characterisation of these surfaces to determine hydrophobicity, chemical composition, topography, level of water adsorption and acrylic bonding ability.

2.3 Materials and Methods

2.3.1 Silicone rubber production

The constituents of the experimental silicone rubber used are shown in Table 2.2. Initially, hydrophobic silica filler was added to a mixture of vinyl terminated silicone polymers (V46 and V21) and a hydride functional silicone cross-linker (5% w/w). These constituents were mixed in a planetary mixer (Kenwood Ltd, Havant, UK) for 2 h to give a final polymeric base containing 20% w/w filler. To cure, 0.15% w/w platinum-cyclovinylmethylsiloxane catalyst was added. The preparation was mixed under vacuum using a Multivac 4 Vacuum Mixer (Degussa Ltd, Darmstadt, Germany) for approximately 2 min, placed in an aluminium mould (2×100×50 mm) which had been pre-coated with Macsil release agent (Polymed Ltd, Cardiff, UK) and finally heated for 1 h at 100°C. The mould had been cleaned prior to use with acetone (Fisher Scientific, Loughborough, UK) and Codent orange solvent remover (Minerva Dental Ltd, Cardiff, UK) to ensure the surfaces were as smooth and reproducible as possible.

2.3.2 Silicone rubber surface treatment

Silicone rubber slabs (20×10×2 mm) were cleaned in hot water containing 2% Lipsol detergent (L.I.P Equipment and Services Ltd, Shipley, UK), rinsed twice with distilled water and once in methylated spirits (Hayman Ltd, Witham, UK), prior to being air dried. Surface modification was performed using argon plasma discharge treatment

Table 2.3: Constituents of silicone rubber produced for surface treatment procedure.

Material	Type	Manufacturer
Polymer mix	Poly(dimethylsiloxane) (vinyl terminated)	ABCR UK Ltd, Manchester, UK
	V46 (high molecular weight)	"
	V21 (low molecular weight)	"
Filler	Hydrophobic silica (812s)	Degussa Ltd, Trostberg, Germany
Catalyst	Platinum-cyclovinylmethylsiloxane complex	ABCR UK Ltd
Cross linker	Methylhydrosilane-dimethylsiloxane	ABCR UK Ltd

followed by exposure to various functional silanes. Plasma treatment was applied to both sides of the silicone rubber using a sputter coater (Edwards, Teddington, UK) modified, to prevent the sputtering of gold, by coating the gold plate with aluminium foil. Samples were plasma treated under 0.2 mbar argon pressure at 5 W for 4 min on each side.

Silane solutions comprised of 2% silane (ABCR, Manchester, UK), 93% absolute ethanol (Fisher Scientific, Loughborough, UK) and 5% distilled water. The eight different silanes used, conveniently designated T1-T8 are shown in Table 2.2. The silane solutions were adjusted to between pH 4.5 and pH 5.5 using acetic acid (Fisher Scientific, UK) or sodium hydroxide (Fisher Scientific, UK). Changes in pH were monitored using a microprocessor pH meter (Hanna Instruments, Leighton Buzzard, UK). The samples were immersed in the silane solutions immediately after plasma treatment for 1 h at room temperature. On removal, the samples were rinsed in absolute ethanol and heated for 1 h at 100°C to cure. The samples were stored at room temperature for at least 24 h
prior to characterisation.

2.3.3 Contact angle determination

Contact angle values for the eight surface treated silicones and the control silicone rubber (n=6) were determined using a computer-linked automated dynamic contact

angle analyser (Cahn instruments Inc, Cerritos, USA). Samples (20×10×2 mm) were constructed and measured accurately using a micrometer and the values entered into the computer. After cleaning (Section 2.3.5), half of the samples were set aside for contact angle testing, whilst the other half were immersed in whole, mixed clarified saliva for 18 h at 37°C (Chapter 3, Section 3.3.3). After removal from saliva, the samples were rinsed in distilled water for 30 s and allowed to dry in air. Both non-saliva coated and saliva coated samples were then tested as described below.

In preparation for contact angle measurements, each sample was positioned above the wetting medium (water) and aligned perpendicular to the liquid. The samples were lowered into the appropriate wetting medium at a speed of 30 μm/sec (Figure 2.2). A graphical image (buoyancy graph) was displayed as the experiment progressed, from which the advancing and receding contact angles were calculated using least squares analysis (Cahn Application Software, Cerritos, USA; Figure 2.3). Differences in contact angles between surface treatments and controls were measured using t-tests and one way analysis of variance (ANOVA; Instat, GraphPad Software Inc, San Diego, USA).

2.3.4 Analysis of Surface Chemistry

The surface chemistry of the silicone rubbers was determined by XPS (VG Escalab 220, East Grinstead, UK; Figure 2.4) using a radiation source that was monochromatisised Al K α (1486.6 eV). Two samples of each surface treatment were tested. Of these two samples, one was a recently treated material (< 1 week) whilst the other had been treated

Figure 2.2: Schematic representation of sample material immersed in wetting medium. Arrow depicts receding contact angle (θ).

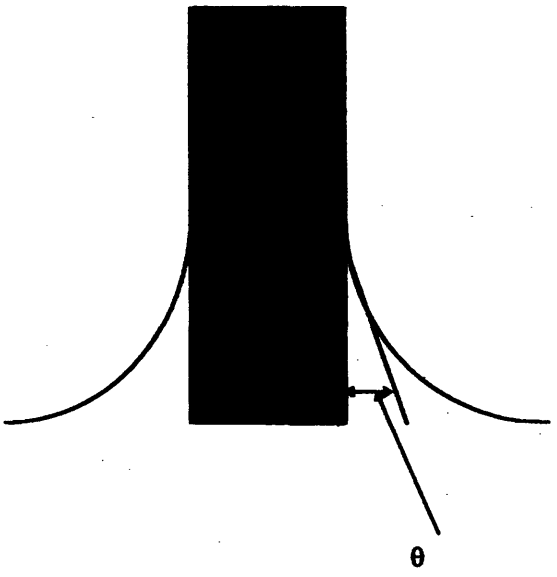


Figure 2.3: Buoyancy graph of force versus sample position for dynamic contact angle analysis. Displayed are least square bars, which are used to calculate the contact angle. Lower line represents surface tension forces as sample is lowered into wetting medium and upper line as sample is removed from wetting medium

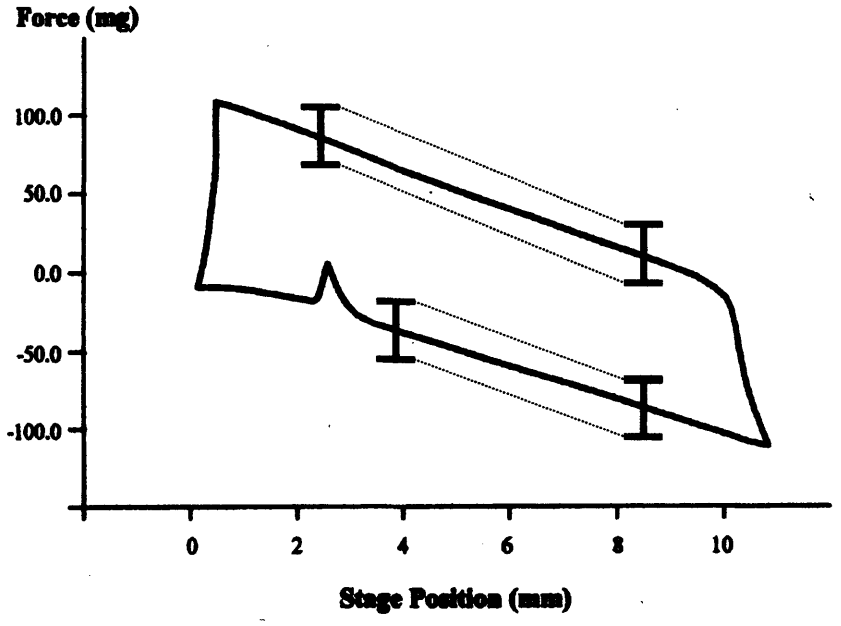
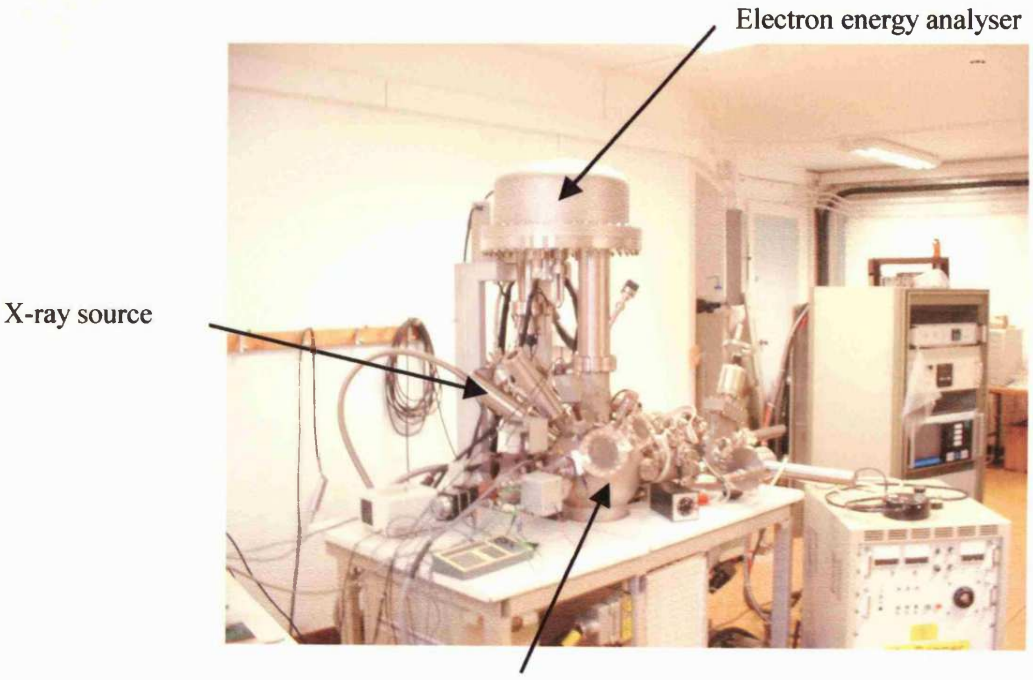


Figure 2.4: XPS machine (VG Escalab 220) for silicone rubber surface characterisation.



6 months previously. These samples were stored at room temperature in petri dishes sealed with parafilm. The latter was included to determine the stability of the surface treatment process over time. Untreated silicone rubber was also tested as a control. The technique was performed at the Physical Chemistry Department at Cardiff University, Cardiff, UK. XPS enabled elemental identification of the first few nanometers depth of the silicone rubber surface.

2.3.5 Analysis of surface roughness

Time limitations led to a representative sample of the treated silicone rubber surfaces being selected for surface roughness analysis. These were T2 (representing hydrophobic treatments), T3, T4 and T5 (representing surfaces that had the same chemical groups as untreated silicone rubber but with altered chain lengths). Surface-treated silicone rubber samples and control silicone rubber (10×10×2 mm) were cleaned for 5 min in 2% lipsol in hot water, followed by rinsing twice in distilled water and finally in methylated spirits. 1 XPS analysis was performed per sample.

Silicone rubber surface topography was characterised by AFM (Dimension™ 3100 Atomic force microscope, Veeco Metrology Group, Santa Barbara, USA). Surfaces were imaged in tapping mode, in air, using silicon cantilevers with a high aspect ratio and typical tip diameter of 10 nm. Before imaging test samples, a 3D reference grid (Veeco Metrology Group) was scanned as a method of calibration. The average imaging

scan rate was 0.5 Hz depending on the roughness of the surface (scan rate was lowered on rougher surfaces to enable improved resolution). Initially, a total of six surface scans (three 50 μm^2 and three 10 μm^2) were taken for each of the treated surfaces and the control silicone rubber. The images produced defined variations in topography using shading according to height (light shading represented areas of increased height compared to areas of darker shading which represented troughs in the surface topography). Average roughness (Ra) measurements and Z-ranges (Rz) were recorded for each scan. Subsequently, a further fifty 1 μm^2 images were produced for each surface treatment of which the Ra and Rz values were also recorded. Comparisons of surface roughness (1 μm^2 scans) were made using t-tests and ANOVA (GraphPad Prism, GraphPad Inc, San Diego, USA).

2.3.6 Water absorption

Surface-treatments T2, T5 and control silicone rubber were measured for up-take of water over a period of 5 months. No contamination of the samples or water was apparent after this time. An increase in the mass of the samples over this period was used as a measure of water absorption. Initially, the samples (n=3) were cleaned according to the protocol in Section 2.3.5. The weight of each sample was recorded to an accuracy of 0.001g, before placing in a desiccator and subsequently re-weighing at regular intervals. Once the weights of the samples were constant, each sample was placed in approximately 15 ml of distilled water in a universal tube. Every two weeks, for a period of 5 months the samples were removed, blotted to remove excess water and

weighed before being deposited in fresh distilled water. After 5 months, the percentage change in weight was calculated and used as a measure of water uptake.

2.3.7 Peel test to assess the bonding of surface-treated silicone rubber to acrylic

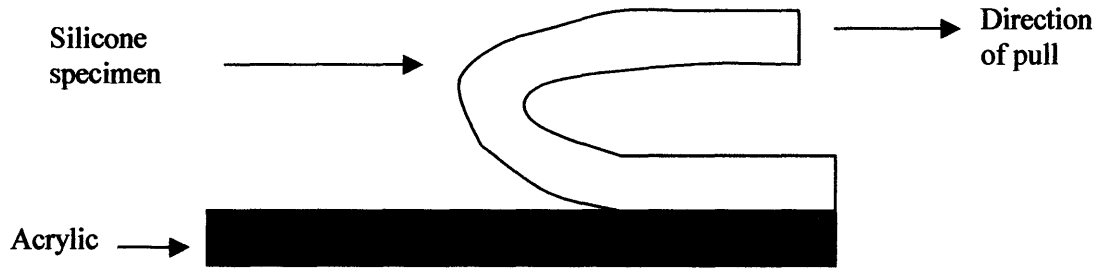
Sample preparation

Using a conventional dental flasking technique, moulds were produced to accommodate a specimen of cold-cure acrylic (2.5×25×75 mm) and an identical sized specimen of silicone rubber positioned directly on top of the acrylic. An acrylic specimen was then cleaned with acetone and allowed to air dry before being partly covered (50×25 mm) in aluminium foil. The foil formed a barrier allowing 25 mm of the acrylic sample to remain un-bonded. The acrylic was then positioned in the mould where the exposed 25 mm of the sample was coated with A-330-G gold platinum primer (Factor II, Lakeside, USA) and heated at 100°C for 30 min. Uncured silicone rubber was poured over the acrylic sample, the mould was then closed and the rubber cured at 100°C for 1 h.

Test Procedure

The bonding strength between the acrylic and silicone rubber was assessed using a Lloyd LR 10K testing machine fitted with a 1 KN load cell (Lloyd Instruments, Fareham, UK). The samples were mounted in self-tightening grips with the un-bonded section of the silicone rubber bent back to give a peeling angle of 180° (Figure 2.5). This section was then pulled at a constant cross-head speed of 20 mm/min. The force per unit width required to peel the silicone from the acrylic was calculated using an equation which takes into account the extension of rubber before commencement of peeling.

Figure 2.5: Schematic diagram of silicone rubber being peeled from acrylic for peel test.



Equation used to calculate peel strength

$$Ps = \frac{F}{W}\left[\frac{1+\lambda}{2} + 1\right]$$

Ps = Peel strength (N/mm)

F = Applied force (N)

W = Width of specimen (mm)

λ = Extension ratio of liner (ratio of the stretched to un-stretched)

2.4 Results

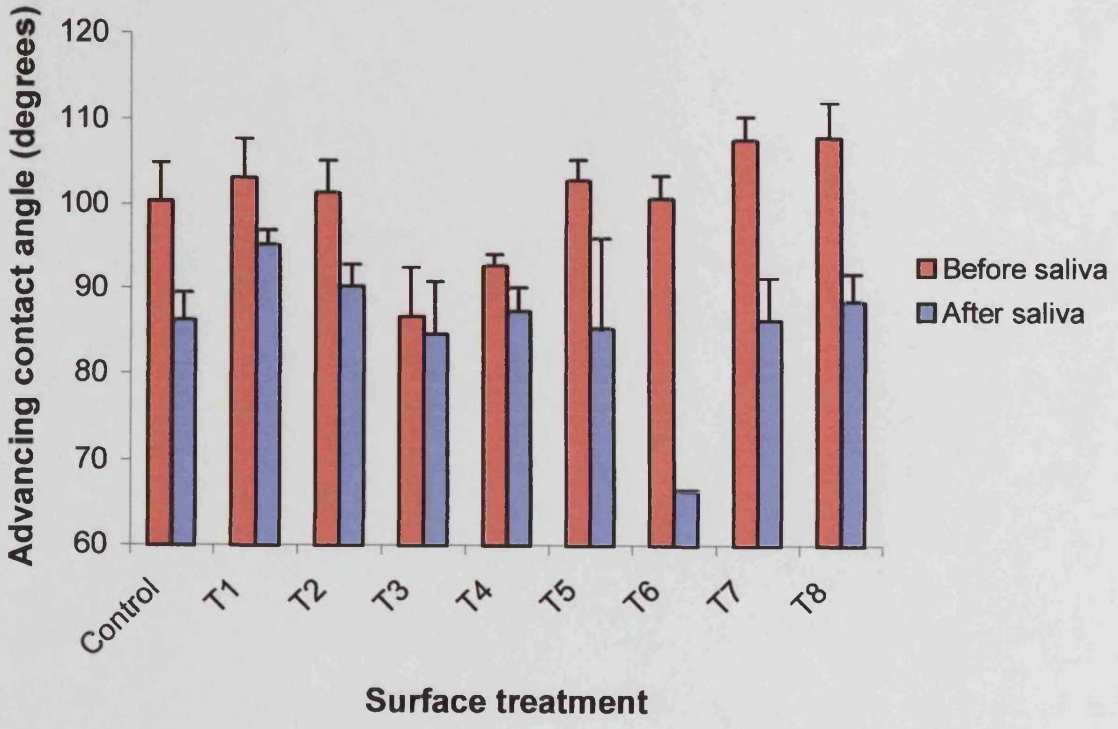
2.4.1 Contact angle determination

Confirmation of the silicone rubber surface treatment was initially assessed using contact angle and XPS data. As shown in Table 2.4, advancing contact angle measurements in water demonstrated a slight degree of alteration in hydrophobicity after surface treatment. Statistically significant differences from the control (100° ± 5, n=12) were only achieved for T3 ($P<0.001$), T4 ($P<0.01$), T7 ($P<0.01$) and T8 ($P<0.01$) treated materials. T3 and T4 treated materials exhibited lower hydrophobicity *i.e.* became more wettable, (advancing contact angles of 86° ± 6, n=6 and 92° ± 2, n=6 respectively). T7 and T8 treated materials showed increased hydrophobicity *i.e.* became less wettable (advancing contact angles of 107° ± 3, n=8 and 109° ± 4, n=8; respectively). Despite demonstrating slightly increased hydrophobicity, the changes were not significant for any of the silanes containing fluorine (T1, T2 and T6). The presence of a salivary conditioning film significantly reduced the contact angle of the control silicone rubber from 100° ± 5 to 86° ± 3 (n=12; $P<0.0001$) and also significantly reduced all surface treatments to between 95° (T1 material) and 66° (T6 material; Figure 2.6 and Table 2.4). T3 treated material was the only material that showed no significant change after pre-coating in saliva. The overall effect of a salivary conditioning film on the silicone rubber was to equilibrate the contact angles to that of the control.

Table 2.4: The advancing contact angles in water of the surface-treated silicone rubber with and without a salivary conditioning film.

Surface treatment	**Contact angle (degrees)**	**Contact angle with a salivary conditioning film (degrees)**
Control	100.44 ± 4.6	86.22 ± 3.22
T1	103.20 ± 4.59	95.13 ± 1.66
T2	101.36 ± 3.71	90.19 ± 2.55
T3	86.66 ± 5.87	84.61 ± 6.23
T4	92.60 ± 1.36	87.19 ± 2.77
T5	102.73 ± 2.46	85.25 ± 10.62
T6	100.60 ± 2.71	66.50 ± 0.00
T7	107.48 ± 2.76	86.23 ± 5.00
T8	107.86 ± 4.13	88.41 ± 3.21

Figure 2.6: Advancing contact angles in water, with and without a salivary conditioning film. Error bars represent standard deviations.



2.4.2 Analysis of surface chemistry using X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy confirmed the presence of the desired chemical groups on the surface-treated silicone rubber. The XPS spectrum for untreated silicone rubber displayed oxygen, carbon and silicone peaks, and when enlarged, no shift was apparent on the carbon peak (Figures 2.7 a and b). The spectra for T1 (data not shown), T2 (Figures 2.8 a and b) and T6 (Figures 2.9 a and b) revealed fluorine peaks, confirming its presence on the silicone rubber surface. Enlarged carbon peaks from these spectra also revealed shifts due the presence of fluorine (Figures 2.8b and 2.9b). A second observation was the absence of chlorine peaks on these spectra. The spectra for T3 (Figures 2.10a and b) and T4 (Figures 2.11a and b) revealed shoulders on the carbon 1(s) peak representing C-O or C=O bonds. XPS was unable to detect differences in chemical composition for T5, T7 and T8, which had surface atoms the same as those on untreated silicone rubber. A further observation was the presence of shoulders on the silicone peaks of the surface-treated silicone rubbers, indicating the presence of silicone atoms (Figure 2.12).

Samples that were tested 6 months after surface treatment maintained the altered surfaces but to a lesser extent. For example, 6 month old T1, T2 and T6 materials demonstrated fluorine peaks approximately half the size of fresh treatments (Figures 2.13 a and b).

Figure 2.7 a: XPS spectrum of control silicone rubber displaying oxygen, carbon and silicon peaks.

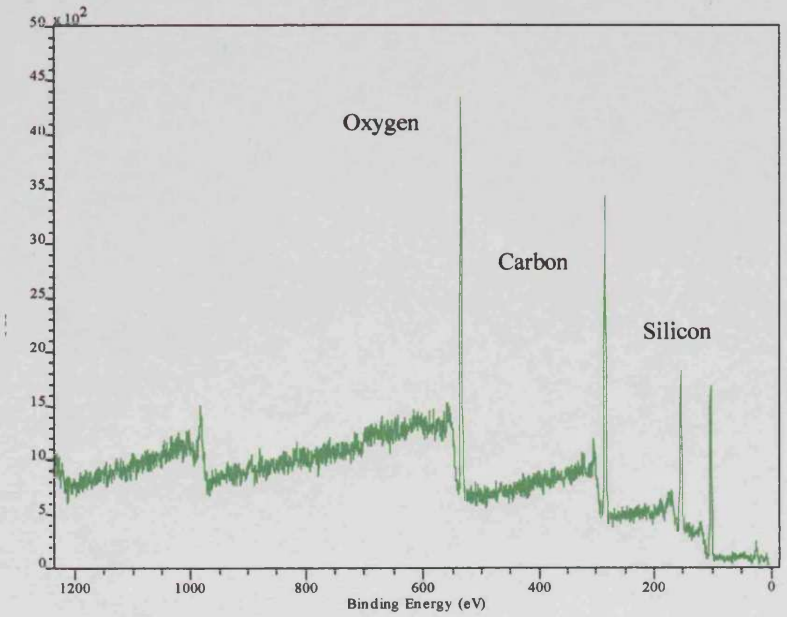


Figure 2.7 b: Enlarged carbon peak on control silicone rubber spectrum.

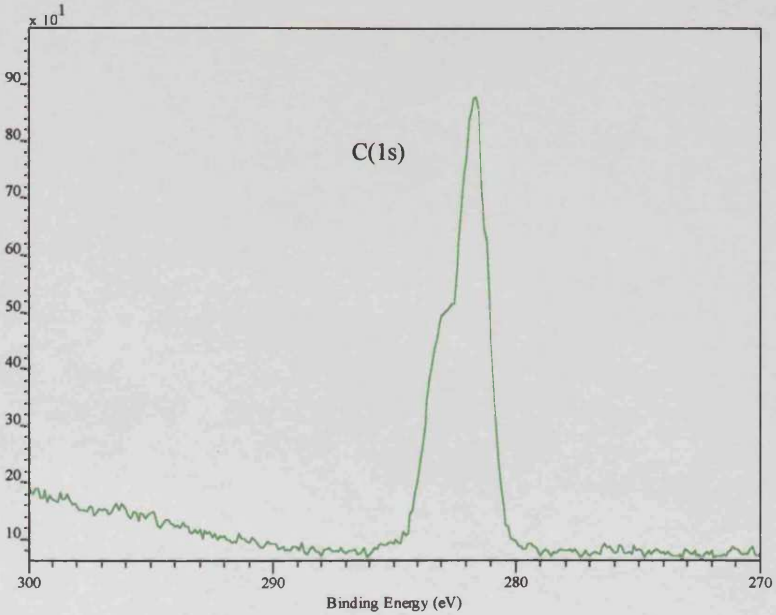


Figure 2.8 a: XPS spectrum of silicone rubber treated with T2 silane, which contains fluorine.

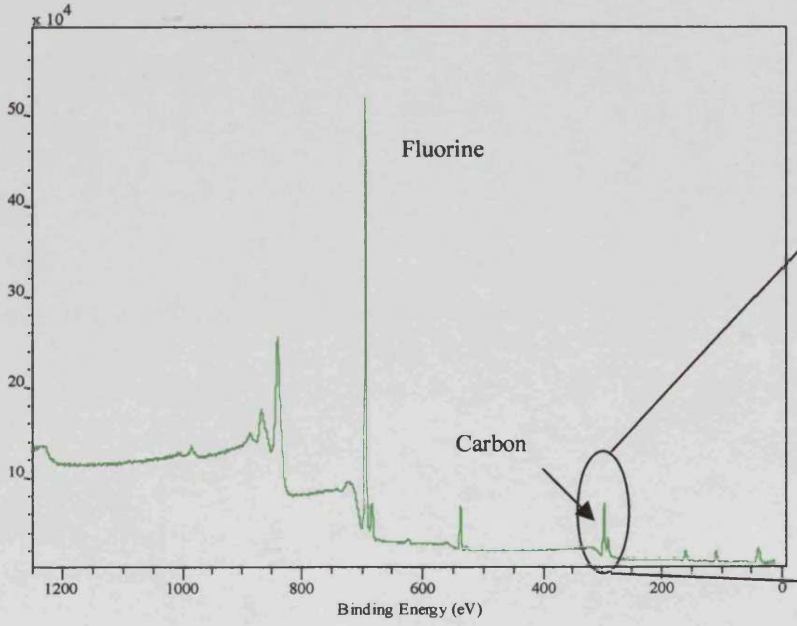


Figure 2.8 b: Enlarged carbon peak displaying the shift caused by the presence of fluorine.

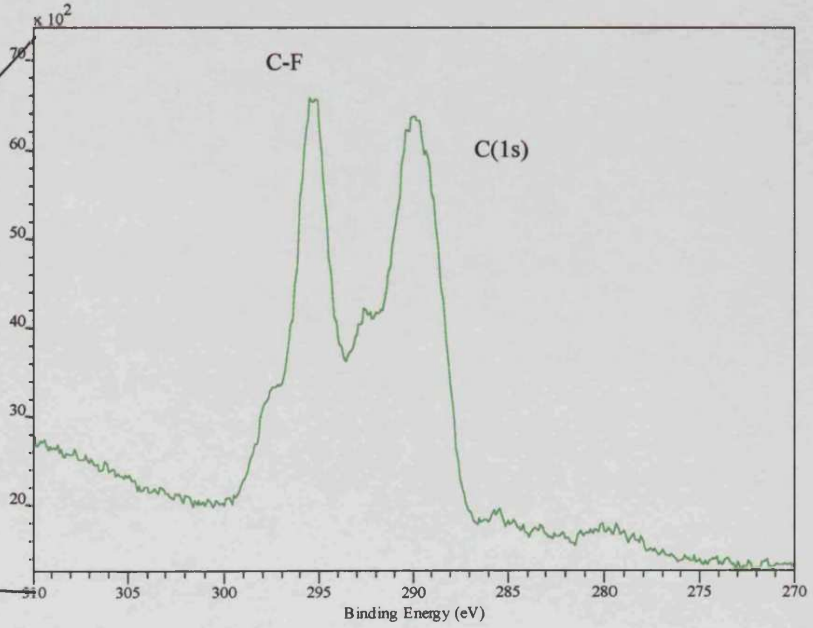


Figure 2.9 a: XPS spectrum of silicone rubber treated with T6 silane.

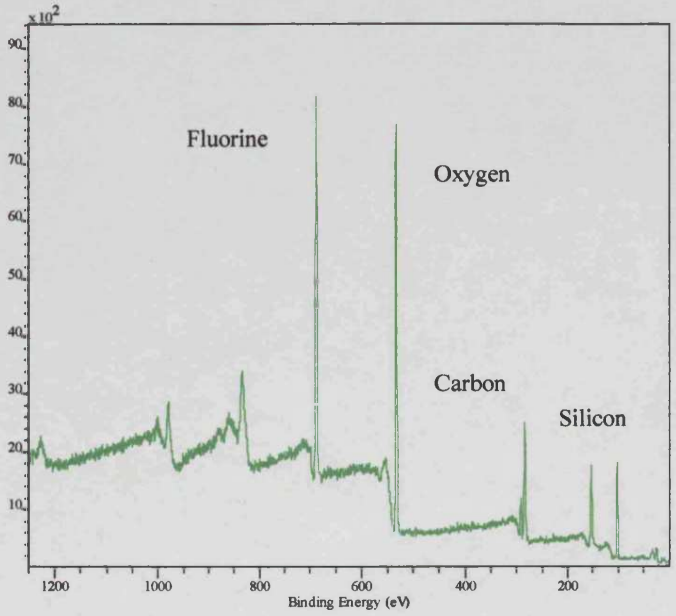


Figure 2.9 b: Enlarged carbon peak from T6 material spectrum displaying the shift in the carbon peak due to the presence of fluorine.

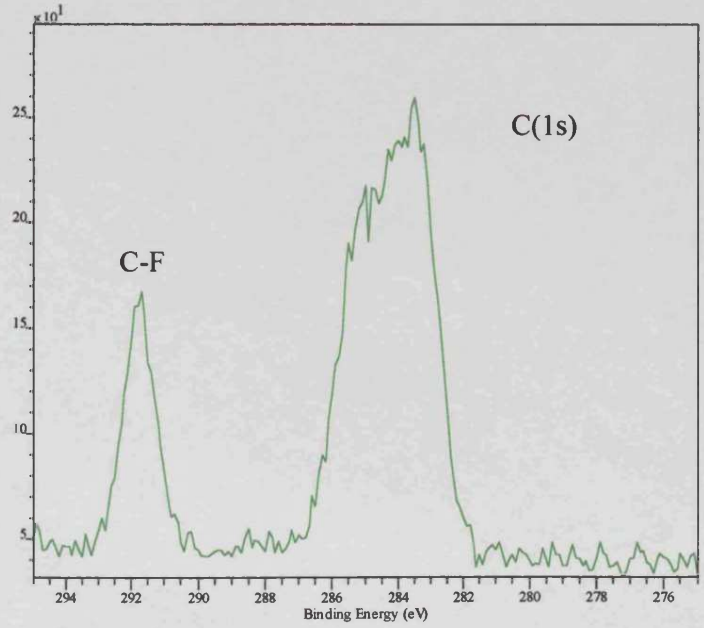


Figure 2.10a: XPS Spectrum of T3 material

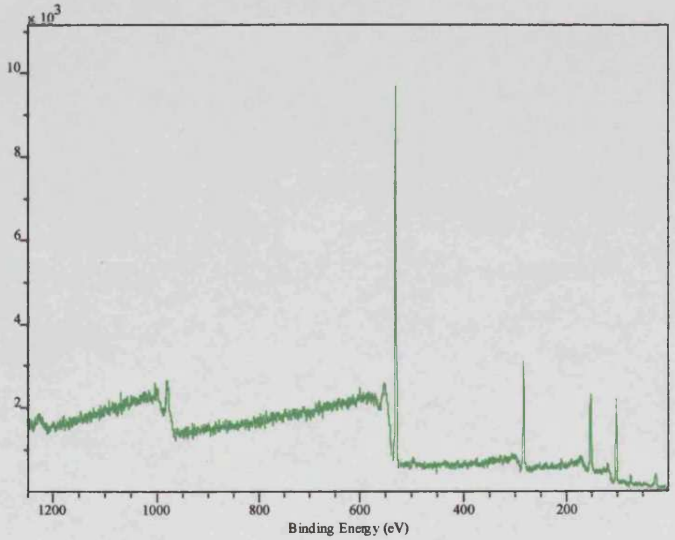


Figure 2.10b: Enlarged carbon 1(s) peak from XPS spectrum of T3 material. A shoulder is apparent on the left of the peak indicating the presence of C=O or C-0.

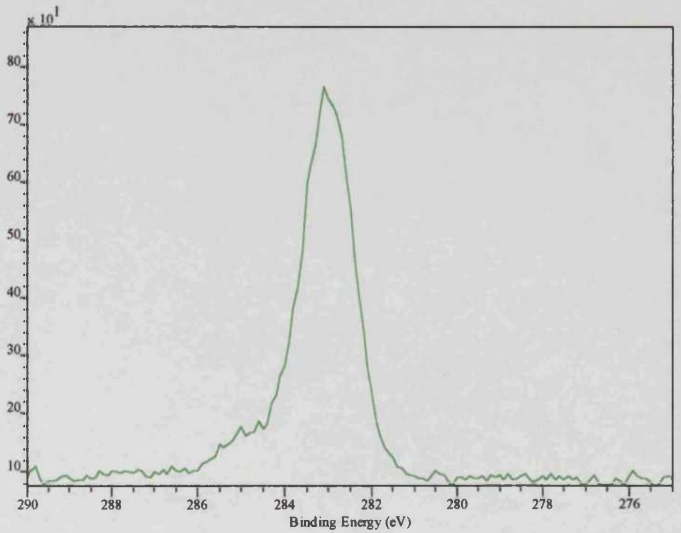


Figure 2.11a: XPS spectrum of T4 material

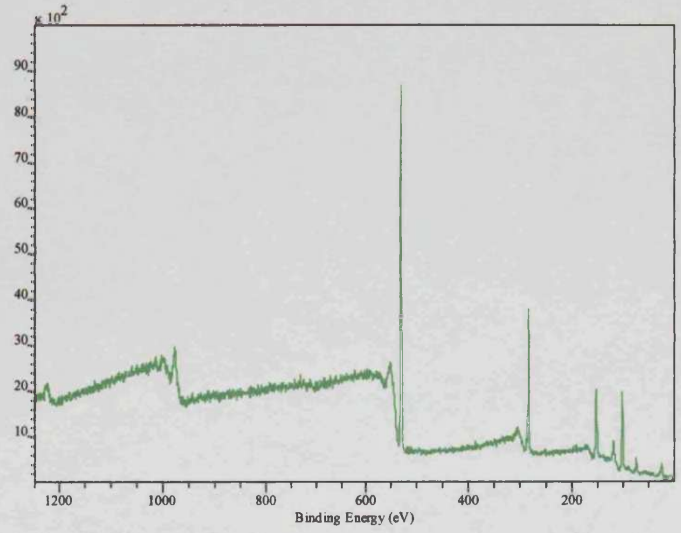


Figure 2.11b: Enlarged carbon 1(s) peak from XPS spectrum of T4 material. A shoulder is apparent on the left of the peak indicating the presence of C=O or C-O.

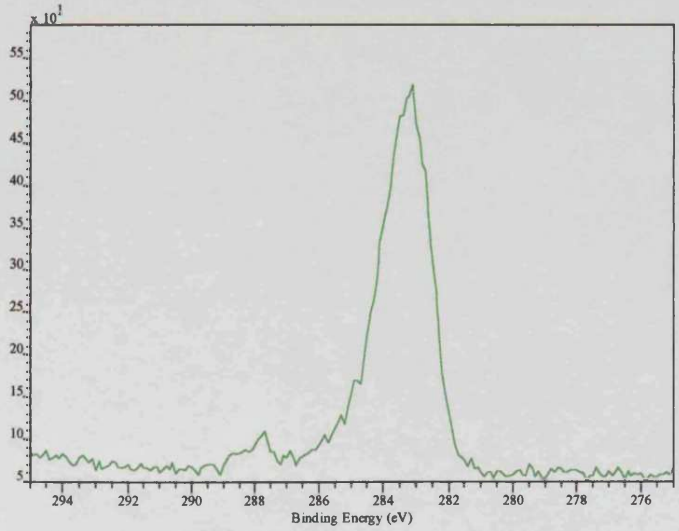


Figure 2.12a: The silicone peak from an XPS spectrum of silicone rubber.

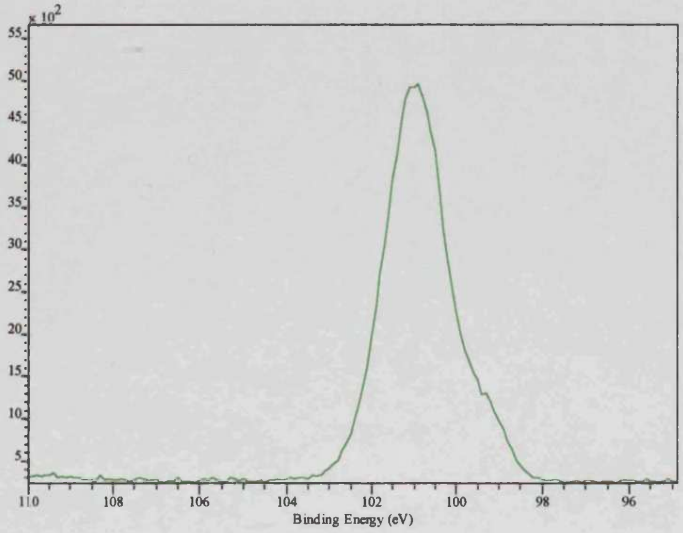


Figure 2.12b: The silicone peak from an XPS spectrum of T2 material. A distinct shoulder is apparent on the right of the peak indicating the presence of silicon.

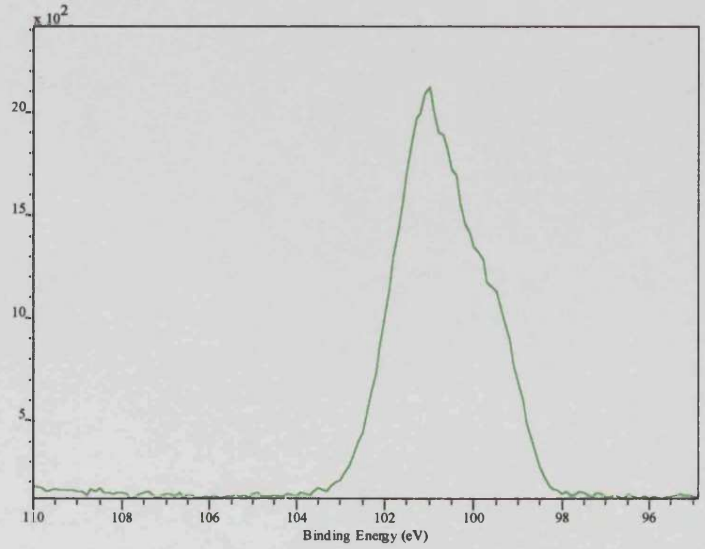
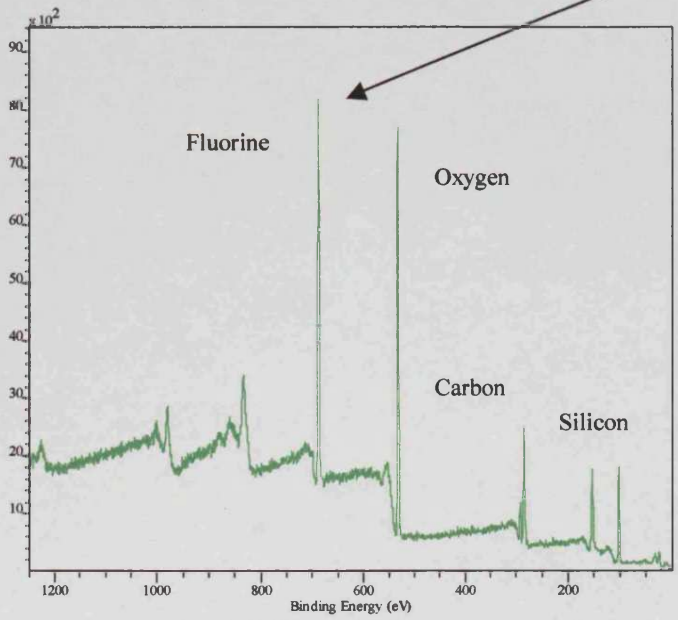
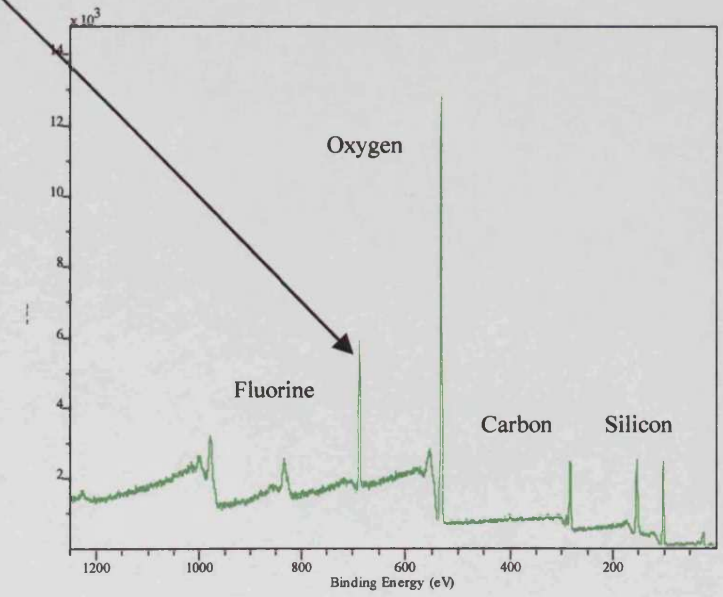


Figure 2.13 a: XPS spectrum of T6 material within one month of being surface-treated.



Decrease in fluorine peak

Figure 2.13 b: XPS spectrum of T6 material, six months after being surface-treated.



2.4.3 Analysis of surface roughness

An AFM image of control silicone rubber of scan size 1 μm^2 is shown in Figure 2.14 and images of treatment T2 compared to the control silicone rubber are shown in Figures 2.15 and 2.16. Qualitative examination of silicone rubber AFM images revealed relatively smooth surfaces, although small surface irregularities were visible on 10 μm^2 and 50 μm^2 images. Images of 1 μm^2 scan size were smooth and flat and displayed very little surface irregularity (Figure 2.14). Visual examination of AFM images revealed no correlative differences in surface topography between the treated surfaces or the control. In fact, topographical features tended to vary between scans on the same test material just as much as between surface treatments. Average roughness (Ra) and Z-range (Rz) values derived from AFM scans enabled quantitative analysis of surface roughness. Images of 10 and 50 μm^2 scan sizes exhibited varied Ra both between surface treatments and between scans on the same material, although in general the roughness was below 50 nm Ra for 10 μm^2 scans and below 160 nm Ra for 50 μm^2 scans (Table 2.5). ANOVAs demonstrated no significant differences in the Ra values between the treated materials and the controls ($P=0.65$ for 10 μm^2 scans and $P=0.53$ for 50 μm^2 scans). Similarly there were no significant differences in Rz measurements ($P=0.83$ for 10 μm^2 images and $P=0.78$ for 50 μm^2 images). Images of 50 μm^2 scan size showed higher Ra values than 10 and 1 μm^2 images due to their larger scan area. The fifty 1 μm^2 images performed on each material revealed significant differences between the Ra of T2 and T3 treated samples ($P<0.001$), T2 and T5 ($P<0.001$) and T3 and T4 ($P<0.001$). T2 and T3 only were significantly different from the control (both $P<0.001$; Table 2.5).

Figure 2.14: AFM image of control silicone rubber using tapping mode (1 μm^2 scan size). Differences in depth of colour represent variation in topographical height (lighter shading indicates structures of increased height).

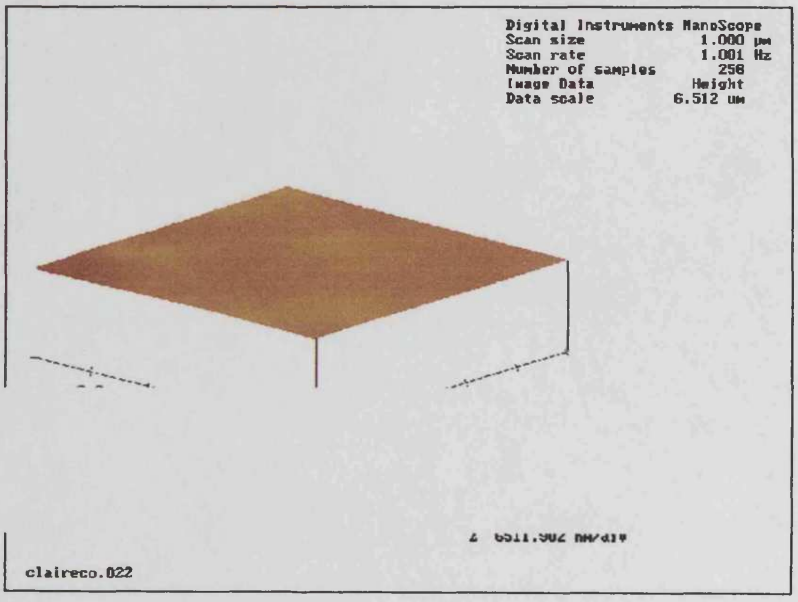


Figure 2.15: AFM image of control silicone rubber using tapping mode (50 μm^2 scan size).

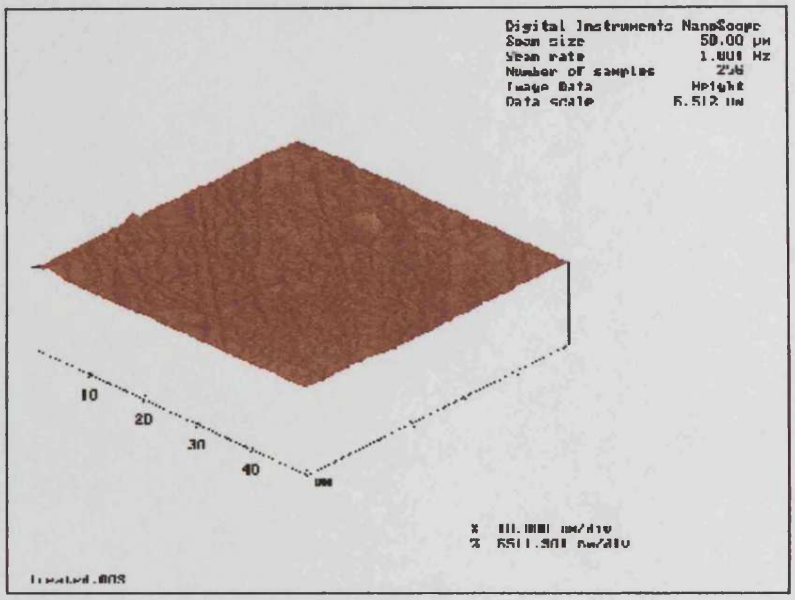


Figure 2.16: AFM image of T2 silicone rubber using tapping mode (50 μm^2 scan size).

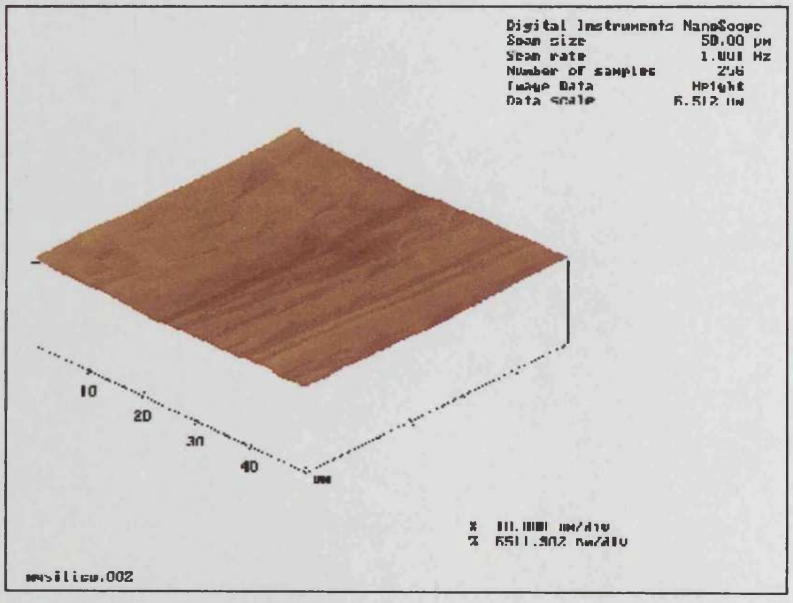


Table 2.5: AFM-derived roughness measurements of surface-treated silicone rubber at three different scan sizes.

Sample	Scan size ± SD					
	1 μm^2 n=50		10 μm^{2*} n=3		50 μm^{2*} n=3	
	Ra (nm)	Rz (nm)	Ra (nm)	Rz (nm)	Ra (nm)	Rz (nm)
Control	4±2	43±24	27±15	307±128	108±51	1617±601
T2	8±3	74±28	50±15	365±151	62±0.4	1211±592
T3	2±1	31±13	32±13	245±156	54±13	576±491
T4	5±3	85±52	36±21	368±86	123±1	1910±906
T5	4±2	44±14	30±13	510±446	98±75	1229±1200

Ra – Average roughness, Rz – Average difference in height between the five highest and five lowest points on a surface.

*No significant difference between surface treatments and control data for these scan sizes.

T3 and T4 displayed significant differences in Ra from the control for 1μm^2 scans (both $P<0.001$).

2.4.4 Water absorption

The surface-treated silicone rubber absorbed less water than control material, although this difference was not statistically significant. Control silicone rubber increased in weight by 0.55% (increase of 0.002 ± 0.0001g on an initial weight of 0.365g) compared with no weight increase for T2 material and an increase of 0.28% (increase of 0.001 ± 0.0001g on an initial weight of 0.359g) for T5 material.

2.4.5 Peel test to assess bonding of surface-treated silicone rubber to acrylic

The force required to peel silicone rubber from acrylic was greater than the tear strength of silicone rubber itself, as the rubber tore before peeling from the acrylic (cohesive failure). The force needed to tear the silicone rubber was 2.21 ± 0.27 N/mm indicating a peel strength higher than this. The bond formed between the acrylic and surface-treated silicone rubber was also stronger than the rubber itself, with forces of 5.45 ± 0.89 N/mm for T2 material and 4.11 ± 0.12 N/mm for T5 material.

2.5 Discussion

Biomaterials are vitally important for patient treatment and management and play a fundamental role in modern medicine. A particularly important biomaterial is silicone rubber, which is used because of its good physical properties and inert nature. However, complications with the use of this material, as well as with other biomaterials, can occur due to colonisation by microorganisms. A biomaterial's non-ablative surface plays a role in this microbial colonisation, which can lead to clinical infection and also impairment of device function. The problem is common and usually leads to the administration of antimicrobials and/or device replacement. Treating patients with device-related infections is time-consuming, inconvenient and infections can be life-threatening.

The aim of the work described in this chapter was to develop a series of novel silicone rubbers with altered surface chemistries for later assessment in microbial adherence assays.

2.5.1 Silicone rubber surface treatment

The surface treatment process activated the silicone rubber surface to enable reaction with silanes. Activation was achieved using argon plasma discharge treatment which removed methyl groups from the silicone rubber surface, which were subsequently replaced by hydroxyl groups on exposure to air. The extent of this activation following

plasma exposure was unclear as was the distribution of surface activation. Previous research has suggested that these parameters are difficult to determine (Ratner, 1992), mainly due to limited surface characterisation techniques. Furthermore, the instability of plasma treated surfaces is a well-known phenomenon (Yializis *et al.*, 2000). Argon plasma treated silicone rubber is not believed to be stable in air (Everaert *et al.*, 1998 a) and the surface hydroxyl groups tend to re-orientate themselves from the surface to the subsurface of the polymer, thus imparting surface hydrophobic recovery. The surface treatment process was thus designed to ensure that newly plasma-treated samples were immersed in the silane solution immediately after treatment. This design step remained constant throughout surface treatments to ensure all samples retained the same level of activation on immersion in the silane solutions. After silane treatment, the surface chemistry was considered stable and permanent, as the activated hydroxyl groups were made stable by bonding with the reactive silane groups. To confirm this, XPS assessment of samples with six-month-old surface treatments was performed and confirmed that the surface treatments lasted for at least six months, although a level of degradation was apparent.

2.5.2 Contact angle determination

Contact angle measurements of the treated surfaces did not reveal any large differences in hydrophobicity. T3 and T4 treated materials, which were treated with hydrophilic silanes were significantly less hydrophobic than the control. The increased hydrophobicity of materials treated with the hydrophobic silanes (T1, T2 and T6) was not statistically significant. Recent studies have stated the difficulty of increasing the hydrophobicity of silicone rubber, which is naturally very hydrophobic (Everaert *et al.*, 1998b). Unexpectedly, T7 and T8 materials had increased contact angles despite not containing fluorine. It is suspected this increase may have been due to batch variations in the silicone polymer mix. The lack of contact angle change did not imply the failure of the surface treatments as the XPS spectra clearly detected the presence of fluorine.

Contact angle measurements on materials coated in a salivary conditioning film suggested that their hydrophobicity decreased. This may have been due to the increased microrugosity of the surface (Radford *et al.*, 1998) or it may have been the effect of polar proteins within the pellicle. Apart from T6 treated material there were no significant differences between the advancing contact angles in water of the treated surfaces and the control materials indicating that the conditioning film neutralised the effects of surface functional groups.

2.5.3 Analysis for surface chemistry using X-ray photoelectron spectroscopy (XPS)

XPS is a sensitive technique for determining surface chemistry and would confirm the presence of the desired functional groups on the silicone rubber surfaces. For material surfaces that incorporated fluorine, as well as the spectra displaying fluorine peaks, there were also shifts in the carbon 1(s) peaks, which indicated the presence of the electronegative fluorine. XPS spectra for T3 and T4 displayed shifts in the carbon 1(s) peaks indicating the presence of oxygen in the form of C-O or C=O bonds, which confirmed the presence of oxygen containing functional groups. XPS spectra for T5, T7 and T8 materials displayed no differences from the control silicone rubber as their surface atoms were the same as those found on untreated silicone rubber. However, all spectra for the surface-treated materials displayed a shoulder on the silicone peak indicating the presence of silicon atoms. Silicon atoms are present in siloxane bonds and therefore confirmed that the functional groups had bonded to the surface. A final observation, was the absence of a chlorine peak on the spectra, which indicated that the silanes were using all possible bonds, as there were no chlorine leaving groups present. XPS was used to assess the longevity of the surface-treatment and revealed that after 6 months, there was a level of degradation of the surface treatments.

2.5.4 Analysis of surface roughness using atomic force microscopy

The uniformity of material surface smoothness is essential as surface roughness is an acknowledged influence on microbial colonisation (Chapter 1, Section 1.5.2). Surface

roughness was monitored using AFM-derived Ra and Rz measurements. Materials surface-treated with T2, T3, T4 and T5 (Table 2.2) were selected for roughness assessment as they provided a representative sample of the surface treatments. Having assessed these four materials it was apparent that chemical treatment did not change the surface topography to an extent that would influence microbial adherence/retention *i.e.* there were no significant differences between the control and treated surfaces for the Ra or Rz measurements on 10 and 50 μm^2 scans.

AFM roughness measurements demonstrated the surface roughness of the silicone rubber was less than 0.15 μm (Ra). Verran and Boyd (2001) proposed three broad categories of surface roughness; macro, micro and nano roughness. Macro roughness has Ra values of ~10 μm, micro of ~1 μm and nano roughness of ~ 0.2 μm. Micro roughness is recognised as the level of roughness suitable for dental implants as demonstrated by Bollen *et al.,* (1997), who showed that an Ra of 0.2 μm was a cut off point below which surface roughness could no longer facilitate microbial adherence. Microbial retention is not believed to occur readily on surfaces with Ra values below 0.2 μm because retention occurs due to surface defects of the same size or slightly larger than the microorganisms. Defects smaller than these are not sufficient to provide microbes with protection from shear forces (Korber *et al.,* 1997). Based on Bollen's study it can be summarised that the Ra values of silicone rubber produced in the present study (below 0.15 μm) should not have facilitated microbial adherence. However, the scan sizes using AFM were very small and it is therefore difficult to gain a representative roughness value for the whole surface. It would be interesting to compare

the AFM roughness results with those obtained using solid stylus or laser profilometry which can measure the roughness of larger areas.

To ensure the reproducibility of surface roughness, the mould was regularly polished to remove scratches and other defects. Despite this, it is inevitable that the surface of the silicone rubber was likely to have several flaws due to scratches and polishing marks on the mould surface which would have been imprinted on to the silicone rubber. These types of defects were hard to avoid as it was difficult to produce a flawless mould. However, the irregularities produced would have most likely been on the macro scale. These macro scale flaws would have been much larger than microorganisms meaning that passive retention of microorganisms would be minimal (Verran *et al.*, 1991). Such flaws were difficult to measure using conventional roughness parameters which generally describe a surface with regular topography (Boulange-Peterman *et al.*, 1997) and do not allow for occasional surface defects.

The Ra values gained from 1 μm^2 scans were irrelevant in respect to microbial adherence that occurs as a result of microbial retention in microbe-sized crevices, as any surface deformations at this level would be too small. The effects of nanoscale roughness on microbial adherence is poorly understood, although must not be ignored, as the type as well as the degree of surface roughness influences adherence (Verran and Boyd, 2001). For example, a novel approach to reducing macrofouling involved the production of a regular mesh structure with 43 and 77μm dimensional roughness. This surface feature greatly reduced the settlement of barnacles whose attachment discs were

of comparable dimensions to the topographical features of the surface. This led to the discs being unable to completely attach (Andersson *et al.*, 1999). Applying this theory to nanoscale roughness, it could be suggested the surfaces could influence the adherence of biomolecules. The AFM $1\mu m^2$ scans revealed some significant differences between the treated surfaces and control. The variations may have been due to the functional groups incorporated onto the surfaces as the measurements were in nanometers. There appeared to be no correlation however, between functional group chain length and surface roughness.

2.5.5 Water absorption

The absorption of water into a polymeric biomaterial is significant due to the swelling it can cause. Swelling can produce distortions in shape which may cause dysfunction of the device and may also lead to weakening of the polymeric structure. Water uptake by a denture-soft lining material can result in a dimensional change creating stress at the liner/denture interface. Stress at this point can potentially cause de-bonding. In respect to microbial colonisation, it has been suggested that a polymer's ability to absorb water may aid the penetration of *C. albicans* and other microorganisms by drawing cells into the material (Taylor and Johnston, in press). Microbial penetration can result in further device dysfunction. All silicone rubbers absorb a certain level of water over time, however, this greatly depends on the hydrophobicity of the filler used (Waters *et al.*, 1996). The silicone rubber used for this project utilised a particularly hydrophobic filler, which explains the low water uptake observed. Water absorption tests were performed

on T2 and T5 materials and also untreated silicone rubber. It was important to test the treated materials as well as the untreated silicone rubber to elucidate if the surface treatment had an influence on water absorption as it could be suggested that the incorporated functional groups may influence the uptake of water. The results demonstrated that the silicone rubber used did not readily absorb water and the surface treatment process had no influence on water uptake into the polymer.

2.5.6 Peel test to assess bonding of surface-treated silicone rubber to acrylic

A strong adhesive bond between silicone rubber and acrylic is essential for the application of silicone rubber as a denture-soft liner. Denture bases are most commonly made from acrylic and need to be able to bond strongly to the soft liner in order to function correctly and prevent patient discomfort. The peel bond strength test was therefore performed in order to assess the effect, if any, of the surface-treatment process on the adhesive bond between the silicone rubber and acrylic. The test performed was introduced by the American Society for Testing and Materials (ASTM) 407, (1986) as a standard method measuring the force necessary to peel the rubber from the acrylic base. The method takes into account both the elastic deformation of the rubber and the adhesive surface energy (Wright, 1982). The peel test results showed the surface treatments did not weaken the bond strength between the acrylic and silicone rubber. In fact, the surface treatments appeared to increase the bond strength.

Conclusions

- Silicone rubber was successfully produced with a level of surface roughness that was deemed to be too low to affect physical microbial retention.
- Argon plasma discharge-exposed silicone rubber reacted successfully with eight different silanes to give materials with altered surface functional groups, as confirmed by XPS.
- Contact angle measurements showed altered hydrophobicities of treated materials and the coating of control and treated silicone rubber with a salivary conditioning film significantly reduced the advancing contact angles in water (apart from T3 material). A salivary conditioning film may have reduced the effect of the underlying surface treatment.
- The surface treatments were proven to be retained for a minimum of 6 months although the extent of surface treatment did appear to reduce with time. Furthermore, the silicone rubber produced did not absorb water over a period of 5 months and surface treatment did not influence water uptake.
- The surface treatment process had no influence on the bonding between acrylic and silicone rubber.

Summary

A range of surface-modified silicone rubbers have been produced, which have been shown by their stability, lack of water uptake and acrylic bonding capability to be suitable biomaterials. The following chapters investigate their potential for inhibiting the adherence of microorganisms.

Chapter 3

Adherence of *Candida* to surface-treated silicone rubber

3.1 Introduction

Candida albicans is a dimorphic fungus, existing as either a blastospore (yeast cell) or as a hypha (filament). Blastospores are spherical in shape and have a specific mode of mitotic cell division known as budding. A hypha is a filamentous tube that arises through germination of a blastospore or through branching of an existing hypha. Hyphae comprise of multiple fungal cell units divided by septa (Figure 3.1).

Candida albicans is the principal opportunistic fungal pathogen of man (Odds, 1988) and is responsible for several human infections, collectively referred to as candidoses (candidosis singular). Candidoses are primarily superficial, occurring on moist mucosal membranes such as in the mouth and vagina. Candidosis can also develop at a variety of other locations including the skin and urinary tract (Table 3.1). Furthermore, systemic candidosis can occur in severely debilitated individuals and these infections are often fatal. Two examples of medical devices which frequently exhibit colonisation by *C. albicans* are voice box prostheses and denture-soft liners.

3.1.1 Voice box prostheses

Candida albicans has the ability to impair the functioning of the voice box prosthesis due to the formation of hyphae which obstruct the valves of the device. The effect of this hyphal obstruction is reduced air-flow through the valves thereby impairing speech formation. A study carried out by Eerenstein *et al.,* (1999) revealed that 95% of explanted voice box prostheses (removed due to dysfunction) were colonised by *Candida* strains. *Candida albicans* was the solitary yeast in 35% of the 183

Figure 3.1: *Candida albicans* yeast cells and hyphae stained with Calcofluor white (original magnification: × 600), courtesy of Dr David Williams, Cardiff Dental School, Cardiff University.

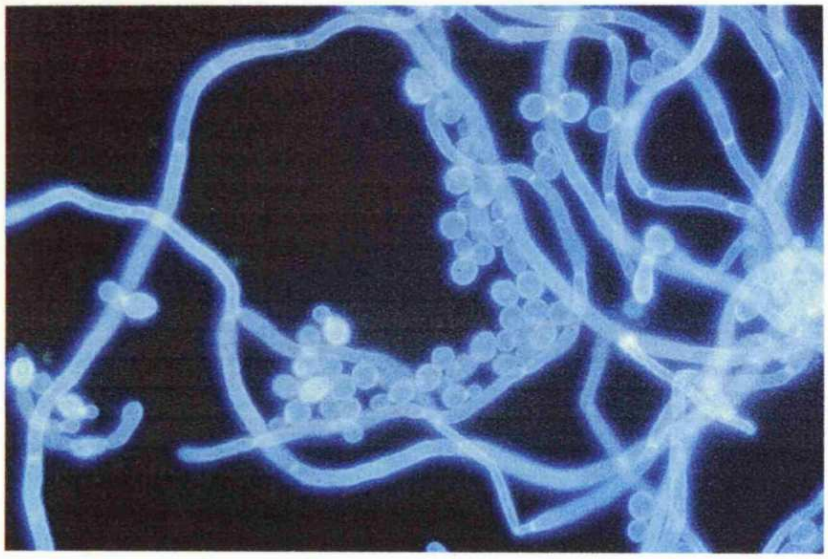


Table 3.1: Typical sites for *Candida* infection and specific candidoses.

Candidosis location	**Disease**
Oropharynx	Acute/chronic pseudomembranous candidosis
	Acute/chronic atrophic (erythmatous) candidosis
	Chronic hyperplastic candidosis
Genitalia	Vulvovaginal candidosis
	Candida balanoposthitis
Skin	*Candida* onychia
	Candida paronychia
Gastrointestinal tract	Oesophageal candidosis
Respiratory tract	Laryngeal candidosis
	Pulmonary candidosis
Urinary tract	*Candida* cystitis
	Candida urethritis
Eye	*Candida* infections of the cornea
	Candida conjunctivitis
Bones and joints	*Candida* arthritis
	Candida osteomyelitis

prostheses examined. The second most frequently encountered species was *Candida tropicalis*, which accounted for 20% of prosthetic colonisation (Eerenstein *et al.*, 1999). Studies have also revealed that in addition to simply adhering to the surface of the prosthesis, *Candida* also demonstrate an ability to penetrate the material itself (Busscher *et al.*, 1994; 1997; Eerenstein *et al.*, 1999). As well as *C. albicans*, some strains of *C. tropicalis* also produce pseudohyphae and have subsequently been found to penetrate silicone rubber voice box prostheses *in vitro* (Busscher *et al.*, 1994). The mechanism behind silicone rubber penetration is uncertain, but enzymatic degradation of the material (Busscher *et al.*, 1994) and penetration mediated by adhesive forces (van Weissenbruch *et al.*, 1997) could play important roles. Eventually, voice box prostheses colonisation by *Candida* species necessitates device replacement, which can be a painful and time-consuming procedure for the patient.

3.1.2 Denture soft-lining material

A denture-soft liner acts as a cushion between the hard denture base and the supporting oral mucosa and is often used in patients with atrophic edentulous alveolar ridges. *Candida albicans* colonisation of dentures is implicated with chronic atrophic (erythematous) candidosis (Figure 1.7 c, Chapter 1; Nikawa, 1993) and studies have shown higher adherence levels to silicone rubber soft liners than to acrylic denture bases (Douglas, 1979).

3.2 Objectives

To determine the level of candidal adherence and penetration to new surface-treated silicone rubber both in the presence and absence of a salivary conditioning film.

3.3 Materials and Methods

3.3.1 *Candida* isolates

Test isolates included *C. albicans* GDH 2346 (Critchley and Douglas, 1984) isolated at Glasgow Dental Hospital, Glasgow, UK, from a patient with CAC), *C. albicans* GRI 681 (laboratory strain, Glasgow Royal Infirmary, Glasgow, UK), *C. tropicalis* MMU1 (clinical isolate, Manchester Metropolitan University, Manchester, UK) and *C. krusei* NCYC 993 (clinical isolate, Manchester Metropolitan University).

Preliminary assays were performed on control silicone rubber in order to optimise yeast adherence. *Candida albicans* GDH 2346 was used and a series of assays performed (Section 3.3.2). Each assay varied either the carbohydrate source in the culture media (glucose and sucrose), material incubation times (30 min, 60 min and 120 min) and material incubation temperatures (37°C and room temperature).

3.3.2 *Candida* adherence assay (static)

The static adherence assay was similar to that described by Williams *et al.,* (1998). *Candida* isolates were cultured separately in yeast nitrogen base (YNB; Becton Dickinson, Microbiology Systems, Sparks, USA) supplemented with 500 mM sucrose at 37°C for 24 h. The resulting yeast growth was harvested by centrifugation (Denley BS400) for 10 min at 1700 g and washed twice in 10 ml of phosphate buffered saline (PBS; 0.01M, pH 7.3; Oxoid Ltd, Basingstoke, UK). The washed

yeast were quantified using an Improved Neubauer haemocytometer (Hawksley, London, UK) and the suspension diluted to a volume of 20 ml containing 1×10^7 yeast/ml. The silicone rubber samples, after being rinsed in 100% ethanol (Fisher Chemicals, UK) and dried in air, were immersed in the prepared yeast suspension within a petri dish and left for 1 h at room temperature. The materials were then rinsed to remove any unattached cells by twice transferring to a petri dish containing fresh PBS and gently agitating for 10 s. On transfer between the dishes, the upper surface of each sample was kept moist by dispensing fresh PBS on to the surface using a Pasteur pipette. The samples were then stained with 0.1% w/v Calcofluor White, (Sigma, Poole, UK) in distilled water and rinsed in PBS to remove excess Calcofluor White. The samples were finally placed on glass microscope slides and overlaid with cover slips.

Adherent yeast were enumerated using a fluorescent microscope (Olympus AX 70, Olympus Optical Ltd, London, UK) attached to a Nikon Digital Camera (DXM 1200) and associated computer. Fluorescently stained yeast were viewed using a DAPI filter ($\lambda_{ex} \sim 440$ nm, $\lambda_{em} \sim 510$ nm) at × 200 magnification (Figures 3.2 a and b). Nine randomly selected fields of view (0.27 mm^2) were digitally captured and the adherent yeast in these images enumerated. Six replicates of each surface treatment were tested on two separate occasions. Statistical analysis using Excel (Microsoft, Redmond, USA) and Instat (GraphPad Software Inc) was by unpaired t-tests and one way analysis of variance (ANOVA).

Figure 3.2: *Candida albicans* stained with Calcofluor White and viewed under fluorescent microscopy using a DAPI filter. a) × 200 original magnification, b) × 600 original magnification.

Figure 3.2a

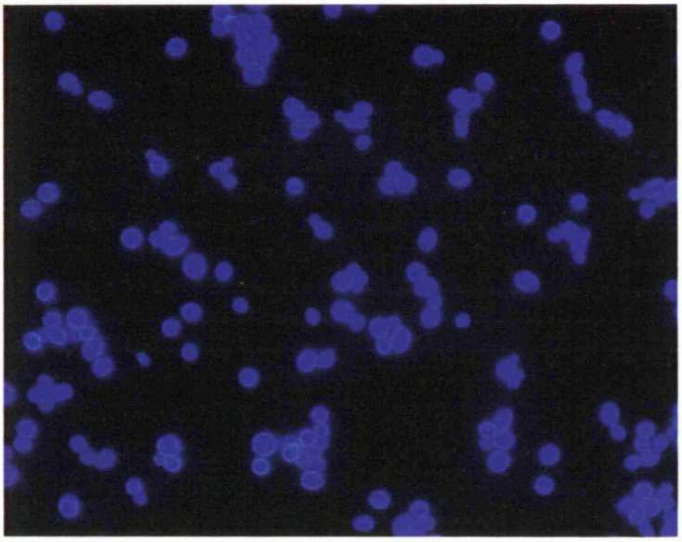
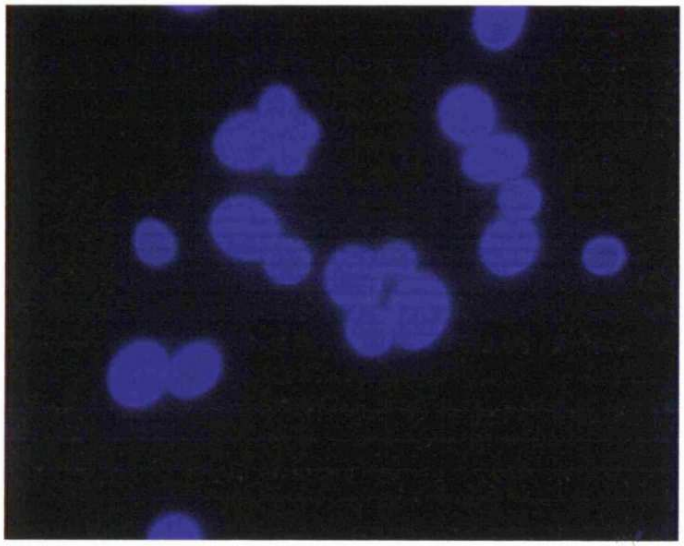


Figure 3.2b



3.3.3 *Candida* adherence assay (static) incorporating a salivary conditioning film

Mixed saliva, stimulated by chewing on parafilm, was collected in universal tubes on ice from 4 male and 4 female volunteers (between 9.30 am and 10.15 am). The saliva was pooled and centrifuged at 40,000 g (RC5C, Sorval Instruments, Du Pont, Stevenage, UK) at 4°C for 30 min. The clarified saliva was aliquoted and frozen (at -20°C) until required.

Silicone rubber samples were rinsed in ethanol, dried in air and placed in T25 tissue culture flasks with 20 ml of saliva. The materials and saliva were incubated for 18 h at 37°C. On removal, the samples were gently rinsed in distilled water using a wash bottle to remove excess saliva. Contact angle measurements (Chapter 2, Section 2.3.3) and the candidal adherence assay (Section 3.3.2) were then performed as described previously.

3.3.4 Candidal penetration of silicone rubber

A candidal penetration assay aimed at determining the in growth of *C. albicans* in to silicone rubber was based on that of Bulad *et al.*, (2004) and Taylor and Johnston, (in press). The assay was undertaken by researchers at the Department of Biological Sciences in Manchester Metropolitan University, UK.

Surface treatment T2 was tested along with control silicone rubber (both produced at Cardiff Dental School, Cardiff, UK; Chapter 2, Sections 2.3.1 and 2.3.2).

3.3.4.1 *Candida* isolates

The in growth of *C. albicans* GDH 2346 (Section 3.3.1) was assessed for surface treatment T2 and control silicone rubber. This isolate was cultured in artificial saliva (Bulad *et al.*, 2004) at 37 ℃ for 18 h.

3.3.4.2 *Candida* penetration assay

Silicone rubber samples (4 control and 16 T2 samples; dimension 10×10×3 mm) were washed in 70% ethanol and twice in sterile distilled water prior to being separately placed in 20ml glass universals containing 10 ml of sterile artificial saliva (Bulad *et al.*, 2004). Each universal was then inoculated with 100 µl of *Candida* culture and placed in an orbital incubator (180 rev/min) at 37℃ for six weeks. Each week, the artificial saliva was replaced with fresh artificial saliva. After the six-week incubation period the samples were removed and immersed in 4% v/v gluteraldehyde in PBS for 2 h. The samples were then transferred through an alcohol series (30-100% ethanol, each for 10 min) before being air dried. Samples were manually sectioned into seven strips (~ 1-2 mm wide) and immersed in crystal violet for 2 min prior to being rinsed in distilled water, acetone (to remove stain from non-yeast structures) and finally distilled water again. Sections were then mounted side on onto glass slides after air drying on filter paper.

Qualitative and quantitative analysis was used to assess levels of candidal penetration into silicone rubber samples. Both hyphal and blastospore penetration were estimated through three depth levels of the silicone rubber. Sections were viewed

using light microscopy (× 400 magnification). Penetration of the material by yeast was graded as 1+ (1-100 cells), 2+ (100-200 cells) and 3+ (>200 cells). Hyphal penetration was similarly graded 1+ (1-5 hyphae), 2+ (5-10 hyphae) and 3+ (>10 hyphae) along the length of the material.

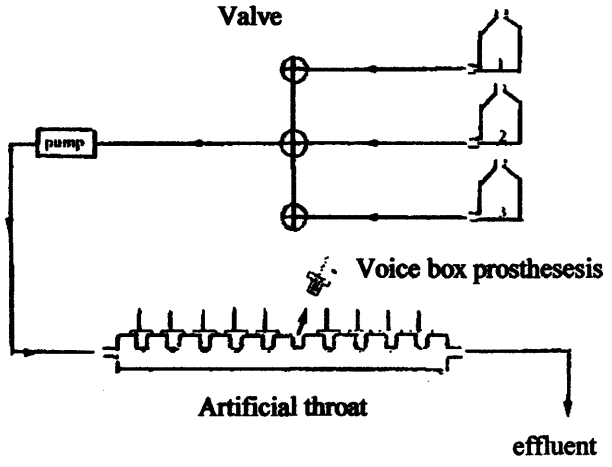
3.3.5 Artificial Throat Method

The artificial throat model assay was designed and performed at the Laboratory of Materia Technica at Groningen University, Groningen, Holland. The method used was adapted from that of Elving *et al.* (2003).

The artificial throat consisted of an elongated Plexiglass flow chamber containing ten insertion plugs, which housed the voice box prostheses (Figure 3.3). The voice prostheses were inserted into the flow-chamber in such a manner that the tracheal side of the prosthesis was exposed to air on the outside of the flow chamber and the oesophageal side was positioned inside the flow chamber (Elving *et al.,* 2003; Leunisse *et al.,* 1999). In this manner, voice prostheses were exposed to microbial cultures, allowing biofilm formation within a closed system.

Groningen Button voice prostheses (Médin Instruments and Supplies, Groningen, Holland) were surface-treated in Cardiff using the method described in Chapter 2, Section 2.3.2. Surface treatments T1, T2, and T4 were tested (four replicates of each). The Groningen Button voice prostheses were made of medical grade silicone rubber (Silastic Q 7-4750, Dow Corning, Midland, USA) and consisted of a shaft with two

Figure 3.3: Schematic diagram of the artificial throat model system



flanges with a semicircular slit of 145° in the hat of the oesophageal flange (Chapter 1, Figure 1.4b).

3.3.5.1 Artificial throat model assay

A mixed culture of bacterial and yeast strains were perfused through the artificial throat model within the flow chamber. All isolates were clinical strains derived from explanted voice prostheses at the University Hospital, Groningen. The isolates used were *C. albicans* GBJ 13/A, *C. tropicalis* GB 9/9, *Stapylococcus aureus* GB 2/1, *Staphylococcus epidermis* GB 9/6, *Streptococcus salivarius* GBJ 52/2A, and *Rothia dentocariosa* GB52/2B.

The bacteria and yeast isolates were co-cultured in 30% brain heart infusion broth (Oxoid, UK) and 70% defined yeast medium (Merk, Darmstadt, Germany; Elving *et al.*, 2003) at 37 °C for up to 5 h. Meanwhile, the surface-treated and control voice prostheses were placed into the inserts of the sterile flow chamber. Inserts not filled with a prosthesis were sealed with plastic plugs providing an enclosed system except for the openings at either end. Within 5 h of incubation, the mixed culture was perfused into the flow cell via the inlet and incubated for 3 days at 36-37°C. On the subsequent 6 days, the artificial throat was flushed three times daily with 250 ml of PBS and allowed to drain. Also, each night, the artificial throat was filled with fresh growth medium (30% brain heart infusion broth, 70% defined yeast medium) for 30 min and allowed to drain overnight (15-17 h). On the eighth day, following a final perfusion with 250 ml of PBS the voice prostheses were removed and the biofilm mechanically removed from the oesophageal flanges. The biofilm samples were

subsequently transferred to reduced transport medium, sonicated (Ultrasonic vibrator, transonic TP690 CLMA) for 60 s and then serially diluted. The diluted suspensions were plated on to MRS (de Man, Rogosa and Sharpe) agar plates for yeast growth (de man *et al.*, 1960) and blood agar plates for bacterial growth. The agars were incubated aerobically at 37°C for 3 days prior to enumeration. Colony forming units (CFUs) were then counted and species identified (Elving *et al.*, 2003).

3.4 Results

3.4.1 *Candida* adherence assay (static)

Table 3.2 presents the adherence data for *Candida* to the surface-treated materials.

It was evident that all the surface treatments (including just argon plasma treatment; Figures 3.4-3.12) significantly reduced *C. albicans* GDH 2346 adherence (in the absence of saliva) compared with controls ($P<0.0001$; Table 3.2). For example, the mean adherence of *C. albicans* GDH 2346 to T5 material was 43 yeast/mm^2 ± 80 (n=5) compared with 2370 yeast/mm^2 ± 1735 (n=50), for the control. There was no correlation between material surface hydrophobicity and candidal adherence (Figure 3.13). Overall, T5 material displayed the greatest reduction in candidal adherence (Table 3.2). There was a significant difference in candidal adherence between T7 and T8 ($P< 0.0001$) materials, with the former appearing to be more conducive to candidal adherence. The adherence of *C. albicans* GRI 681 was greatly reduced on all samples compared with GDH 2346 (Figure 3.14 and Table 3.2).

The surface hydrophobicity of the materials was significantly reduced ($P<0.0001$) following pre-coating with saliva (Chapter 2, Figure 2.5). All the treated materials exhibited a similar decrease in hydrophobicity on the addition of the salivary conditioning film. Pre-treatment of materials with saliva resulted in an elevated level of *C. albicans* GDH 2346 adherence (Table 3.2, Figure 3.15). For example, adherence on T1 material without saliva was 64 yeast/mm^2 ± 22 compared to 2576 yeast/mm^2 ± 721 in the presence of a conditioning film.

Figure 3.4: *C. albicans* adhering to control silicone rubber (× 200 original magnification).

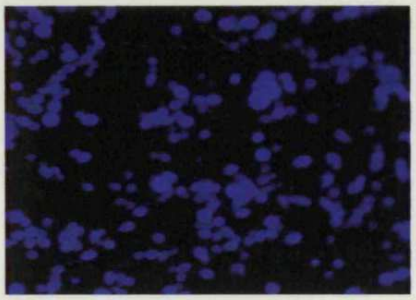


Figure 3.5: *C. albicans* adhering to T1 material.

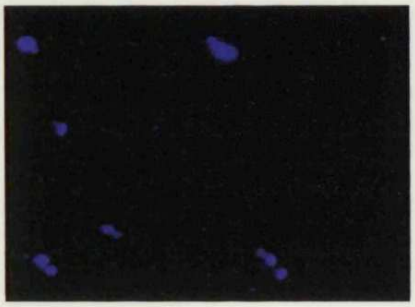


Figure 3.6: *C. albicans* adhering to T2 material.

Figure 3.7: *C. albicans* adhering to T3 material.

Figure 3.8: *C. albicans* adhering to T4 material.

Figure 3.9: *C. albicans* adhering to T5 material.

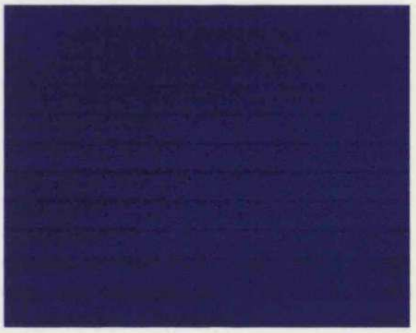


Figure 3.10: *C. albicans* adhering to T6 material.

Table 3.2: ***Candida*** **adherence to surface-treated, argon plasma-treated (without silane treatment) and control silicone rubber with and without saliva. Displayed are mean adherence values and standard deviations.**

Candida	Treatment	Adherence without saliva (yeast/mm^2)	Adherence with saliva (yeast/mm^2)
C. albicans GDH			
2346	Control	2370 ± 1735	1596 ± 1118
	T1	64 ± 22 *(P<0.0001)*	2576 ± 721 *(P<0.001)*
	T2	88 ± 180 *(P<0.001)*	1137 ± 804 *ns*
	T3	568 ± 511 *(P<0.0001)*	2819 ± 1948 *(P<0.001)*
	T4	382 ± 128 *(P<0.0001)*	837 ± 681 *(P<0.001)*
	T5	43 ± 80 *(P<0.0001)*	513 ± 438 *(P<0.0001)*
	T6	126 ± 64 *(P<0.0001)*	1373 ± 933 *ns*
	Control*	631 ± 384	
	Argon plasma	10 ± 27 *(P<0.0001)*	
C. albicans GRI 681			
	Control**	6 ± 3	
	T1	4 ± 4	
	T4	3 ± 3	
	T5	2 ± 2	
C. tropicalis MMU1			
	Control	9106 ± 3561	49 ± 31
	T2	3842 ± 3346 *(P<0.001)*	136 ± 130 *ns*
	T5	2300 ± 120 *(P<0.001)*	92 ± 85 *ns*
C. krusei NCYC 993			
	Control	11847 ± 3792	
	T2	10512 ± 4439 *ns*	

P values in first column compare control and treated materials in the absence of saliva. P values in second column compare control and treated materials in the presence of saliva.

* Control tested during assay performed on argon-plasma treated material.
** Values not large enough for statistical analysis

Figure 3.11: Mean adherence (yeast/mm^2) of *C. albicans* GDH 2346 to surface treated silicone rubber. Error bars represent standard deviations.

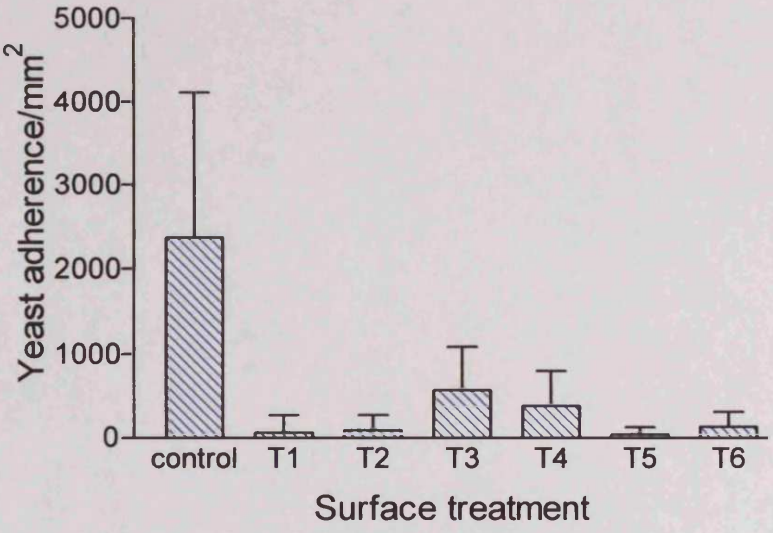


Figure 3.12: Mean adherence (yeast/mm^2) of *C. albicans* GDH 2346 to argon plasma-treated silicone rubber and control silicone rubber. Error bars represent standard deviations.

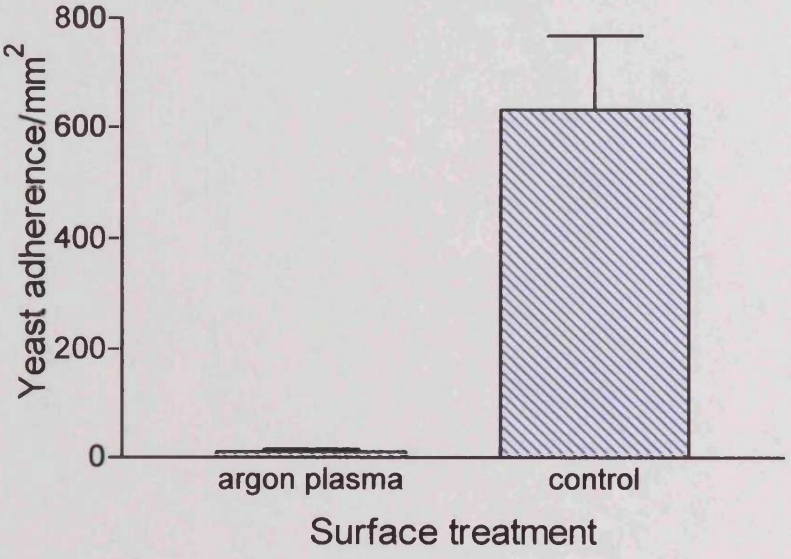


Figure 3.13: Relationship between surface hydrophobicity (advancing contact angle in water) and the adherence of *C. albicans* GDH 2346 (yeast/mm^2). Error bars represent standard deviations.

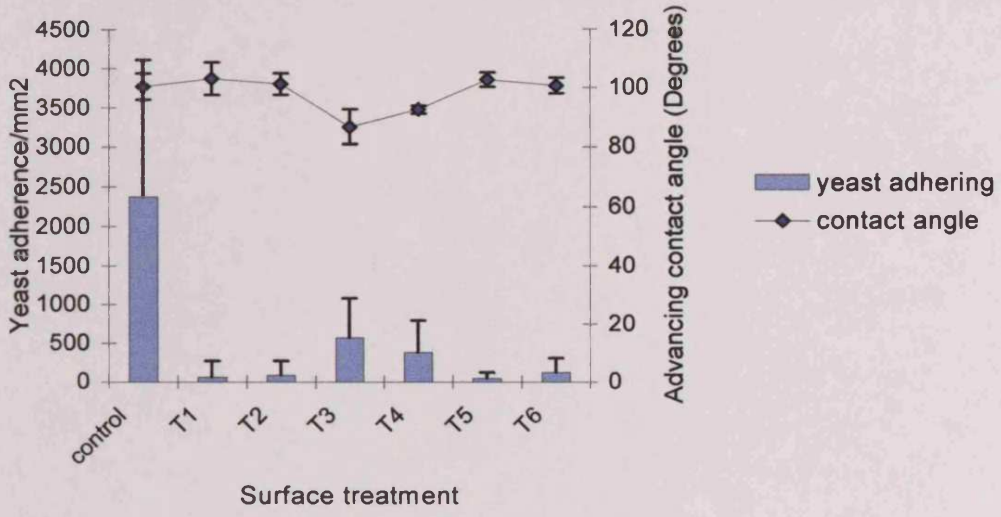
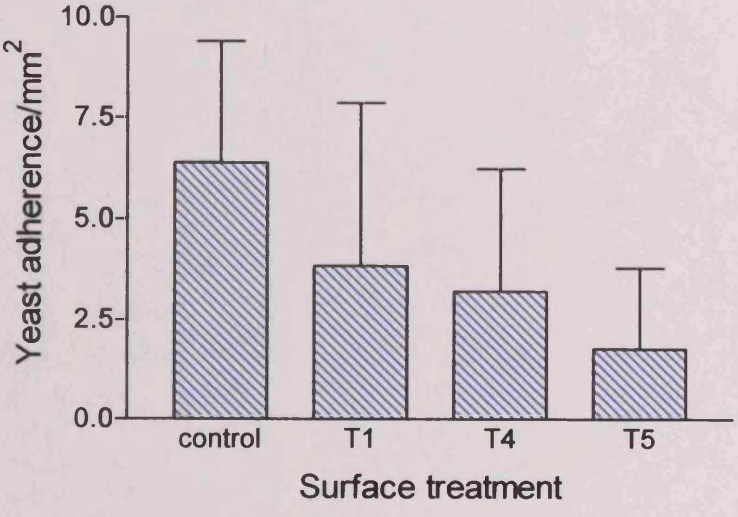


Figure 3.14: Mean adherence (yeast/mm^2) of *C. albicans* GRI 681 (non-adherent strain) to surface-treated silicone rubber. Error bars represent standard deviations.



Interestingly, an equivalent increase was not evident with control materials. The change in *C. albicans* GDH 2346 adherence as a result of the saliva coating was not consistent for the materials. The presence of a salivary conditioning film on materials T1 and T3 resulted in a significantly higher level of *C. albicans* GDH 2346 adherence, which was in contrast to materials T4 and T5. The previously encountered significant difference ($P<0.0001$) between T7 and T8 materials was no longer evident following material coating with saliva ($P=0.17$; Table 3.2, Figure 3.16).

To summarise, the effect of the salivary conditioning film on surface-treated materials was to decrease hydrophobicity and increase yeast adherence. In the case of materials T4 and T5, reduced Candidal adherence compared with controls was still evident.

3.4.2 *Candida tropicalis* adherence assay (static)

Initial *C. tropicalis* adherence was higher to both control and surface-treated materials (Figure 3.17) when compared to *C. albicans* GDH 2346. The adherence of *C. tropicalis* to control material was nearly three times greater than that of *C. albicans* GDH 2346 to control material. However, T2 and T5 materials displayed significantly lower *C. tropicalis* adherence (both $P<0.001$) than controls. A 42% reduction was observed on T2 material and a 25% reduction on T5 material. This reduction was not as great as that with *C. albicans* GDH 2346. The addition of a salivary conditioning film dramatically reduced *C. tropicalis* adherence levels on both the control and surface treated materials.

Figure 3.15: Mean adherence (yeast/mm^2) of *C. albicans* GDH 2346 to surface-treated and control silicone rubber both with and without the presence of a salivary conditioning film. Error bars represent standard deviations.

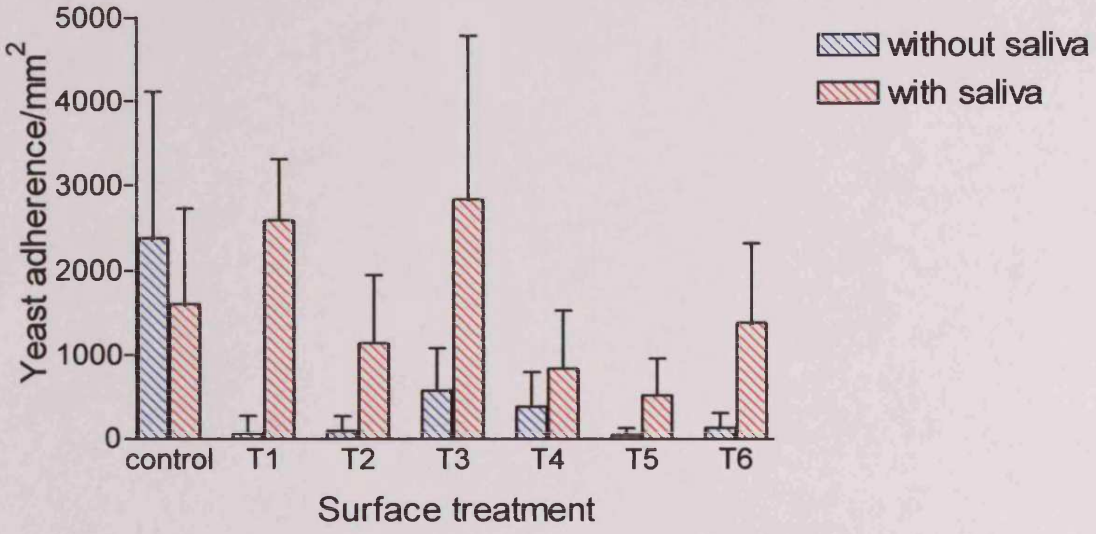
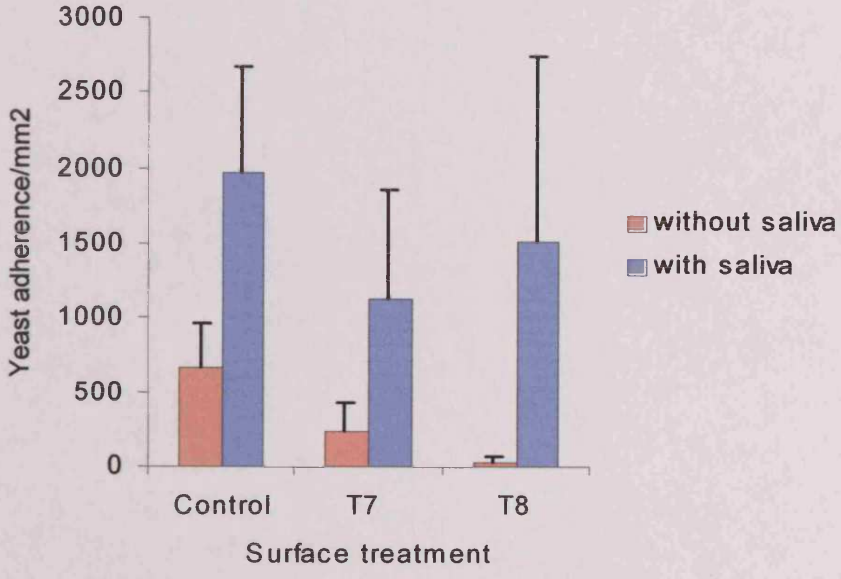


Figure 3.16: Mean adherence (yeast/mm^2) of *C. albicans* GDH 2346 to surface-treated and control material both with and without the presence of a salivary conditioning film. Error bars represent standard deviations.



The control adherence reduced from 9106 yeast/mm^2 ± 3561 (n=18) to 49 yeast/mm^2 ± 31 (n=9) on coating with saliva. Adherence to T2 material was 3842 yeast/mm^2 ± 3346 (n=18) without a salivary conditioning film, which dropped to 136 yeast/mm^2 ± 130 (n=18) with a conditioning film. Similar results were evident for T5 material.

3.4.3 *Candida krusei* adherence assay (static)

Candida krusei adherence was assessed on material T2 and untreated material. Adherence was particularly high on both the control and surface-treated silicone rubber (Figure 3.18). The mean control level of adherence was 11847 yeast/mm^2 ± 3792 (n=9) and the adherence on T2 material was 10512 yeast/mm^2 ± 4438 (n=9). No significant difference between the control and treated material was evident.

3.4.4 *Candida* penetration assay

The frequency and graded quantity of blastospore penetration decreased from the surface to the inner levels of the samples (Table 3.3). Trails of blastospores from the inner levels of the sections were observed in both T2 and control material. However, T2 material was generally cleaner than the control with very few surface defects and only a few yeast observed. A very thin biofilm was observed on the control material with sparse areas of blastopsores. Hyphal penetration was observed on both materials but to a lesser extent in T2 material. In fact, only one section was found to have hyphae and this was at level 1, *i.e.* the surface of the material whereas the control displayed hyphal penetration at level three (Table 3.4).

Figure 3.17: Mean adherence (yeast/mm^2) of *C. tropicalis* to T2 and T5 materials and control material. Error bars represent standard deviations.

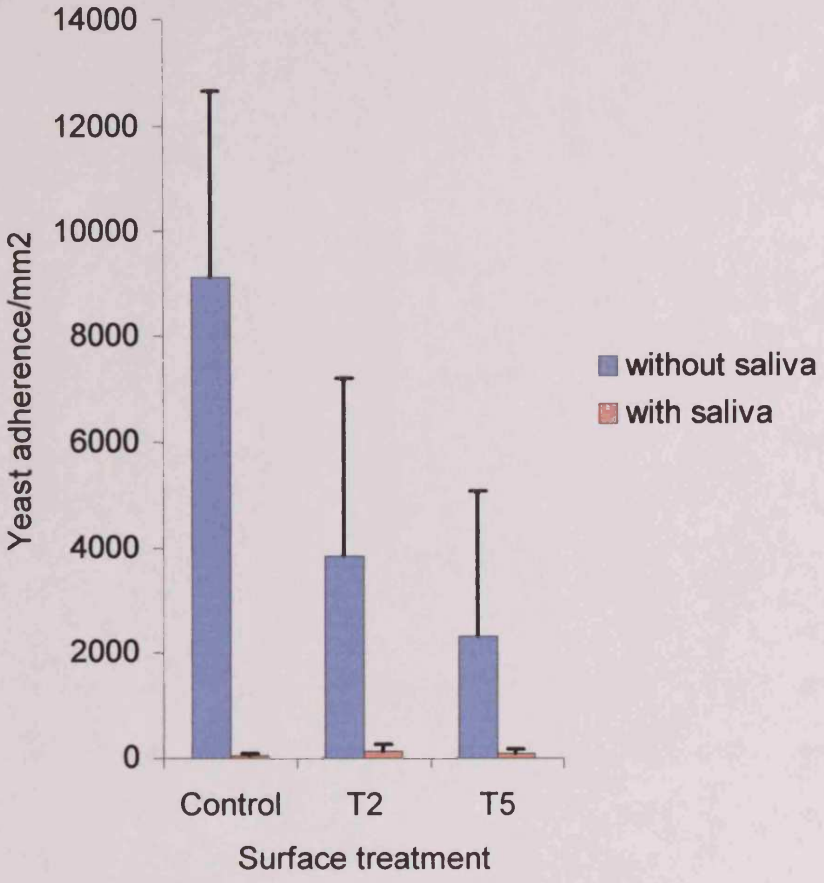


Figure 3.18: Mean adherence (yeast/mm^2) of *C. krusei* to T2 material and control silicone rubber. Error bars represent standard deviations.

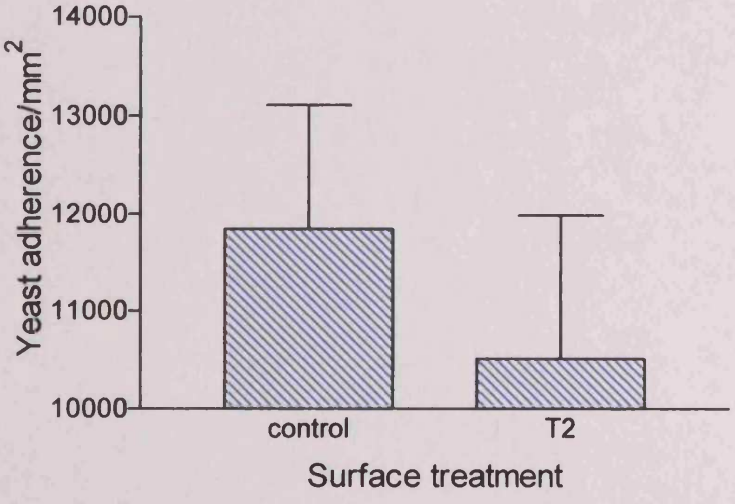


Table 3.3: Penetration of *C. albicans* GDH 2346 cells into silicone rubber. T2 material was tested against control silicone rubber. Displayed are the number of cells found penetrated in different levels of the silicone rubber, with level three being the deepest.

	Level 1				Level 2				Level 3			
	3+	2+	1+	-	3+	2+	1+	-	3+	2+	1+	-
T2	-	3	8	2	-	-	1	1	-	-	-	20
Control	-	3	16	1	-	1	9	7	-	-	2	16

Data courtesy of Manchester Metropolitan University

Table 3.4: Penetration of *C. albicans* GDH 2346 hyphae into silicone rubber. T2 material was tested against control silicone rubber. Displayed are the number of hyphae found penetrated in different levels of the silicone.

	Level 1		Level 2		Level 3	
	2+	1+	2+	1+	2+	1+
T2	-	-	-	-	-	-
Control	-	1	-	3	-	1

Data courtesy of Manchester Metropolitan University

3.4.5 Artificial throat model assay

A reduced candidal biofilm was detected on the three surface-treated voice prostheses compared with controls. T2 treated voice prosthesis displayed the lowest level of yeast biofilm formation, with between $1 \times 10^5 - 1.5 \times 10^5$ CFUs occurring on MRS agar plates. T1 treated voice prostheses exhibited ~2×10^5 CFUs and T5 prostheses, ~4×10^5 CFUs. Two control prostheses were tested in each artificial throat model, one at the front (by the inlet tube) of the model and one at the back (by the outlet tube). The control at the front of the model had ~1.4×10^6 CFUs and the control at the back of the model had ~6×10^5 CFUs.

3.5 Discussion

Silicone rubber is a widely used biomaterial for clinical applications as it is biocompatible, resilient and readily available. However, problems can arise due to microbial adherence on the silicone rubber surface. This is a common problem with many medical devices, including voice box prostheses and denture soft-liners, which frequently become colonised by *Candida.* The colonisation leads to biofilm formation, which can cause clinical infection and destruction of the medical device. Due to their dimorphic nature, *C. albicans* and *C. tropicalis* play an important role in device destruction, as hyphae and pseudohyphae can block the valves of a voice box prosthesis and also invade the silicone rubber, disrupting its structure.

The aim of these experiments was to assess a range of surface-modified silicone rubbers (Chapter 2) to determine their affinity for a series of *Candida* isolates. The modified surfaces were produced with the aim of reducing the adherence of *Candida.* The treatment process used altered the surface chemistry of silicone rubber without the changing the bulk properties (Chapter 2, Section 2.3.2).

The present chapter reports on a series of adherence assays employed to assess the affinity of *Candida* to the novel silicone rubber surfaces.

3.5.1 *Candida* adherence assay (static)

The choice of *Candida* isolates used for the adherence assays at the Dental School in Cardiff was based upon their previously reported adherence capabilities to a variety

of surfaces. *Candida albicans* GDH 2346 has been identified as an adherent strain (Critchley and Douglas, 1987), particularly to epithelial cells (McCourtie and Douglas, 1984), acrylic (Williams *et al.,* 1998) and silicone rubber (Verran and Maryan, 1997). In contrast, *C. albicans* GRI 681 exhibits low adherence (Critchley and Douglas, 1985; Waters *et al.,* 1997) and was included to aid comparison and to ensure that the new materials did not promote the adherence of previously poorly adhering strains. In addition, other *Candida* species, namely *C. tropicalis* MMU1 and *C. krusei* NCYC 993 were included in the study. To date, *C. albicans* is the most commonly isolated *Candida* species colonising the oral cavities of healthy adults (Holmes *et al.,* 1995) and is also the most frequent cause of chronic erythematous candidosis (Budtz-Jørgenson, 1974). However, recent reports have indicated an increase in the incidence of infection caused by other *Candida* species. For example, a study reported that non-*albicans Candida* species were emerging as relatively common causes of oropharyngeal candidosis in head-and-neck cancer patients (Dahiya *et al.,* 2003). This is an important concern in patients who have voice box prostheses, as these devices are primarily used following laryngeal cancer. *Candida tropicalis,* in particular, has been implicated in causing increased resistance to air flow in voice box prostheses (Elving *et al.,* 2003). *Candida krusei* has also been identified as an emerging pathogen (Samaranayake *et al.,* 1994), particularly in systemic infections in bone marrow recipients (McQuillen *et al.,* 1991). To make the study relevant to clinical practice it was therefore important to include these two species of *Candida* in the present study.

Past studies have used a variety of methods to evaluate the adherence of microbes to materials. Many have incorporated parallel plate flow chambers (Chapter 5, Section

5.2) where a constant flow of microbial suspension is passed over the sample material for several hours or even days. Such a flow-through method is ideal for testing materials used in areas of the body where a steady fluid flow is encountered. Typical examples would be the lumens of intravenous or urinary catheters. The initial adherence assay used in this study was based on that of Williams *et al.*, (1998) and was developed to mimic salivary flow surrounding prostheses in the oral/oropharyngeal cavities. Within these cavities, medical devices are in contact with slow flowing or static saliva. Therefore, in the present assay, the silicone rubber samples were allowed to stand in a static suspension of *Candida.* This was similar to the assay carried out by Nikawa *et al.*, (1989), where the adherence of *Candida* was assessed to surface-modified glass slides. The slides were incubated vertically in a beaker containing the yeast suspension. Suspending the samples vertically helped to prevent microbial sedimentation which is important as microbes adhered in this manner could be mistaken as adhering. The assay employed in this study could have potentially allowed sedimentation of *Candida* on the silicone rubber surfaces but the rinsing process performed prior to enumeration was aimed at ensuring all non-adhered cells were removed. In the past, enumeration of yeasts adhering to materials was commonly performed by removal of the material from the microbial suspension and subsequent staining of yeast with stains such as acridine orange (Waters *et al.*, 1997). While this approach permits visualisation of the yeast, removal of the material from the liquid allows the occurrence of an air-surface interface, which promotes aggregation of cells on the material surface. The aggregation is believed to be caused by surface tension forces exerted across the surface of the material as it dries (Williams *et al.*, 1998) and the resulting aggregated yeast tend to be difficult to enumerate. Williams *et al.*, (1998) developed a novel technique for enumeration

using an inverted microscope, which enabled yeast adhered to acrylic to be enumerated whilst submerged in buffer, therefore avoiding an air-surface interface. This method was attempted in the present study but was deemed unsuitable when using silicone rubber because of small imperfections in the material such as trapped air, which could be mistaken for yeast cells. Aggregation of yeast in this assay was avoided by providing a constant flow of buffer to the material surface during transferral between petri dishes. Surface drying whilst enumerating was prevented by overlaying the material surface with cover-slips to retain moisture.

Adhered yeast were visualised by fluorescent microscopy, after staining with Calcofluor White; a fluorescent stain that binds to chitin in the yeast cell wall (Nicholas *et al.,* 1994). The fluorescent stain ensured the yeast were readily distinguishable from imperfections in the silicone rubber.

The adherence assay was optimised to obtain a high initial yeast adherence to the control material. This was achieved by varying the carbohydrate source and its concentration within the culture media and also by altering the incubation temperature. The assay which yielded the highest level of adherence utilised a culture media supplemented with 500 mM sucrose and an incubation temperature of 37°C (results not shown).

The second adherence assay involved subjecting the silicone rubber to mixed whole saliva for 18 h to produce a salivary conditioning film, a method adapted from Samaranayake *et al.,* (1980). The composition of saliva is variable and exhibits intra and inter variation in individuals (Edgar, 1992). To minimise the effects of such

variations, pooled saliva was obtained from several subjects of both sexes at the same time each day. The saliva used was stimulated in order to provide a mixture of saliva from all the salivary glands. The presence of a salivary conditioning film on the materials may have been more relevant to candidal adherence on denture-soft lining materials than to voice box prostheses, as recipients of prostheses are likely to be receiving radiotherapy, chemotherapy or systemic steroid therapy to treat laryngeal cancer (Zegarelli, 1993). These medical treatments, particularly irradiation to the head and neck area are strongly associated with xerostomia (lack of salivary secretion) and hyposalivation (abnormal reduced salivary flow rate; Wright, 1987). In one study, these two disorders were evident in 77% of patients with advanced malignancy (Jobbins *et al.,* 1992). The reduced saliva within this patient group is likely to mean voice prostheses are not coated in a salivary conditioning film although there are treatments for the disorders such as mucin-containing oral sprays (salivary substitute) and pilocarpine hydrochloride (salivary stimulant). However, even with the administration of these agents, patients may still suffer from inadequate salivary production and this, in itself is a known predisposing factor for opportunistic candidal infections (Chaushu *et al.,* 2000).

3.5.2 *Candida* adherence to surface-treated silicone rubber

The adherence of *C. albicans* GDH 2346, GRI 681 and *C. tropicalis* MMU1 were reduced on the surface-treated silicone rubber. There was heterogeneity in the adherence of the isolates to both controls and surface treatments. *Candida tropicalis* was the most adherent followed by *C. albicans* GDH 2346 and finally *C. albicans* GRI 681. *Candida tropicalis* was only tested against T2 and T5 materials as these

had previously exhibited inhibition of *C. albicans* adherence. Adherence of *C. krusei* to surface-treated materials was extremely high and displayed no reduction compared to the control. This was unexpected as *C. krusei* is thought to be one of the least pathogenic and less adherent *Candida* species. However, *C. krusei* is clinically relevant due to its recent increased detection in candidosis infections (Samaranayake *et al.,* 1994) and inherent resistance to certain azole antifungals. The cell wall constituents of microorganisms vary between strains and species and *C. tropicalis* is known to have different surface characteristics to *C. albicans.* For example, *C. tropicalis* yeast tend to be more hydrophobic than *C. albicans.* (Busscher *et al.,* 1997; Minagi *et al.,* 1986), which may account for their greater adherence to silicone rubber, which is hydrophobic. Similarly, Samaranayake *et al.,* (1994) found that in a study of 20 *C. krusei* strains, the cell surface of *C. krusei* was also more hydrophobic than that of *C. albicans.* Others have reported similar results (Minagi *et al.,* 1985) although in these studies only one strain of *C. krusei* was tested. The study by Samaranayake *et al.,* (1994) also noted that the adherence of *C. krusei* to both HeLa cells and acrylic was much higher than that of *C. albicans* (mean adherence of *C. krusei* to acrylic was 203 yeast/unit area compared to 19 yeast/unit area for *C. albicans*). The study concluded that cell surface hydrophobicity is related to adherence; a hydrophobic cell will adhere to a hydrophobic surface. However, whilst adherence is a key event in the pathogenesis of microorganisms (Masuoka and Hazen, 1997), other virulence mechanisms obviously contribute to the pathogenesis of *Candida* thereby maintaining the elevated position of *C. albicans* over *C. krusei* and *C. tropicalis* in the pathogenic hierarchy of the genus.

The adherence of *C. albicans* GDH 2346 was also tested on argon plasma treated silicone rubber with no subsequent silane treatment. Plasma treatment of silicone rubber produced unstable (Chapter 2, Section 2.5.2) polar groups (hydroxyl groups) on the surface but did not add any extra functional groups. Assays revealed inhibition of candidal adherence on these surfaces.

3.5.2.1 Determining adherence mechanisms to surface-treated silicone rubber

Since all materials, irrespective of hydrophobicity, exhibited reduced adherence of *Candida* (apart from *C. krusei;* Section 3.4.3), the reduction was unlikely to be linked to the hydrophobicity of the material surfaces. Past research has indicated relationships between these parameters. However, changes in hydrophobicity after surface treatment were limited. In fact, only T3 and T4 materials had significantly altered contact angles *i.e.* they were less hydrophobic, and studies have shown that fairly large changes in hydrophobicity are required to influence microbial adherence (Everaert *et al.,* 1998b). This may in part explain the inhibition of *Candida* adherence to the argon plasma-treated surfaces (with no silane treatment), which displayed large decreases in hydrophobicity (Everaert *et al.,* 1998a). It is difficult to increase the hydrophobicity of silicone rubber because of its very high initial hydrophobicity (Everaert *et al.,* 1998b). For example, Silver *et al.,* (1995), only managed to increase the water contact angles of silicone rubber by 5° from 108.5° to 113.6° upon fluoro-alkyl chemisorption after oxygen plasma treatment.

Three other possible surface characteristics that were changed following silane treatment may be important in reducing adherence. These characteristics were: 1)

the actual surface chemical groupings, 2) functional group-chain length and 3) increased mobility of the surfaces due to the presence of siloxane bonds. It is unlikely that surface chemical groups were responsible for inhibition as three of the eight surface treatments (T5, T7 and T8) contained the same chemistry as untreated silicone rubber, (*i.e.* carbon and hydrogen groupings), yet still caused a reduction in adherence. Alteration of chemical chain length has previously been considered as a possible way of reducing adherence (Everaert *et al.,* 1998b). It may be that long-chain functional groups provide a physical barrier which prevent microorganisms from getting close enough to the surface for non-specific adherence mechanisms (*e.g.* electrostatic interactions) to occur. The inclusion of T7 and T8 treated material was to specifically determine whether long chain functional groups were responsible for reducing candidal adherence. Both T7 and T8 materials had the same surface chemistry as silicone rubber, meaning that variables related to chemical composition of the functional groups were absent. T7 (hexyltrichlorosilane) material constituted an extensive chain of methyl groups attached to the trichlorosilane (nine methyl groups in total) creating a surface with long-chain functional groups. T8 (methyltrichlorosilane) material, contained trichlorosilane with only one methyl group attached resulting in a surface with very short-chain functional groups. The adherence results for both materials showed reduced candidal adherence. Furthermore, T8 treated material actually demonstrated significantly less adherence than T7 treated material. It was therefore demonstrated that long-chain functional groups were not responsible for inhibiting candidal adherence. However, it is important to note the hypothesis of Everaert *et al.,* (1998b), stating that silanes polymerise and accumulate into extensive structures (Chapter 2, Section 2.1.1) on the silicone rubber surface. If this is correct, the shorter chain length of T8 material may

not be an important factor, but rather the ability of the coatings to produce extensive surface structures.

The third surface characteristic that may have been the cause of reduced adherence, was increased surface mobility due to the presence of siloxane bonds. The presence of siloxane bonds was the only common feature amongst all the treatments suggesting that their presence was the cause of reduced candidal adherence. The siloxane bonds have a high degree of freedom for reorientation in an aqueous environment indicating a level of surface mobility (Everaert *et al.*, 1998b). Their mechanism of action may have been to provide a constantly mobile surface, that prevented microbes from adhering long enough for specific binding to occur. The siloxane bonds may have also provided steric hindrance to receptor and ligand bonds.

3.5.2.2 *Candida* adherence assay (static) incorporating a salivary conditioning film

Previous research on the effect of saliva on adherence is contradictory. Busscher *et al.*, (1997) found that a salivary conditioning film reduced the rate of yeast and bacterial adherence to silicone rubber. In contrast, Nikawa *et al.*, (1993), demonstrated higher yeast adherence to saliva-coated denture lining materials. Later studies have also showed that saliva and serum conditioning films not only enhance colonisation of *C. albicans* but also promote hyphal invasion of the colonised material (Nikawa *et al.*, 1997a).

A salivary conditioning film present on the surface-treated silicone rubber in this study, increased candidal adherence, (with the exception of *C. tropicalis,* Section

3.4.2). However, T4 and T5 materials still had significantly reduced candidal adherence compared to the saliva-coated control. T2 and T6 also showed reduced adherence although this was not statistically significant. This does not mean however, that such materials should be discounted and further examination in the future may be warranted.

These results demonstrate that in the presence of saliva, the surface treatments behaved differently from one another in relation to candidal adherence. This again, would appear to discount surface hydrophobicity as a cause of altered adherence since the reductions in hydrophobicity observed due to coating in saliva were similar with all the surface treatments. This contradicts the findings of others (Klotz *et al.,* 1985; Nikawa *et al.,* 1989; Odds, 1988; Satou *et al.,* 1988) who state that alteration in the hydrophobicity of inert surfaces is the cause of changes in microbial adhesion. Nikawa *et al.,* (1992) found no correlation between the contact angles and zeta potentials of protein-immobilized glass and the level of candidal adherence. Based on these findings it was concluded that specific interactions between the surfaces and the cells may be important.

Since material surface hydrophobicity was not hugely altered by surface treatment, it has been excluded as a possible cause of altered adherence in this particular study. Two explanations for the altered candidal adherence have become apparent. Firstly, it is known that saliva and other conditioning films mask the effect of the underlying surface (Taylor *et al.,* 1998). It is possible therefore that the surface treatments were adsorbing the constituents of the salivary conditioning film to varying degrees. For example, T1 material, which exhibited high levels of candidal adherence in the

presence of a conditioning film, may have adsorbed large quantities of conditioning film constituents with the result of negating the previous inhibitory effect of the surface. This is in contrast to T5 material, which showed less adherence, suggesting a lower adsorption of conditioning film constituents. This hypothesis is consistent with the findings of Arkles *et al.,* (1980) who stated that octadecyl functional groups (as contained on T5 treated material) incorporated onto surfaces inhibit the adsorption of protein. If this hypothesis is correct, further work would be needed to determine why the surface treatments were adsorbing salivary constituents to different extents.

A slightly different explanation for the variations in candidal adherence between treated surfaces can be drawn from work by Everaert *et al.,* (1998b) and Pratt-Terpstra *et al.,* (1989). It has been proposed that fluoro-alkyl chemisorption on silicone rubber may inhibit adherence in the presence of saliva by either selective protein adsorption, or conformational changes of the adsorbed proteins. There are many protein components within saliva (Chapter 1, Section 1.5.4). Some of these (including proline-rich proteins (PRPs) and mucins) are known to constitute a salivary conditioning film on dental enamel and oral medical devices. PRPs are believed to promote the adherence of *C. albicans* to surfaces (O'Sullivan *et al.,* 1997), whilst proteins such as histatins have a candidacidal domain and inhibit adherence. The presence of a salivary conditioning film is therefore likely to alter microbial adherence and this could be the cause of enhanced microbial adherence on the treated surfaces. For reduced microbial adherence to occur, it could be argued that either a lower concentration of proteins that bind *Candida* or a higher concentration of proteins inhibitory to *Candida,* need to be present within the

salivary pellicle on the surface-treated material. For example, T1 material which had a high candidal adherence level could have had a low affinity for anti-candidal adhering proteins such as histatins or, conversely a high affinity for *Candida* adherence-enhancing proteins such as PRPs.

Conformational changes of the adsorbed proteins on surface-treated materials offers another possible explanation. This hypothesis was put forward by Lassen *et al.*, (1994), who suggested that salivary proteins could adsorb to hydrophobic surfaces in an unnatural conformation which is unfavourable to bacterial adherence. Similar ideas have been postulated by Pratt-Terpstra *et al.*, (1989), who proposed that conformational changes in proteins arise due to the surface chemistry of materials.

In the presence of saliva, the adherence of *C. tropicalis* decreased dramatically, which may partly explain why it is less pathogenic than *C. albicans.* Nikawa *et al.*, (1992), assessed the adherence of *C. albicans* and *C. tropicalis* to mucin, saliva and fibrinogen-immobilised glass slides. Whilst their results illustrated the adherence of *C. albicans* to mucin and saliva-immobilised glass slides, *C. tropicalis* bound only to the fibrinogen-immobilised slide. The study concluded that *C. tropicalis* does not bind to salivary proteins. This indicates the reduction in adherence must have been due to the salivary proteins covering the surface of the silicone rubber.

3.5.3 Penetration and artificial throat model assays.

The penetration assay demonstrated that T2 material was more effective at resisting yeast and hyphal penetration than control silicone rubber, thus supporting the results

of the adhesion assays. Blastospores cannot actively penetrate silicone material and it has been suggested that the silicone rubber imbibes the culture solution, swelling the material and drawing in *Candida* cells (Taylor and Johnston, in press). The surface-treated silicone rubber displayed very little water absorption as illustrated in the water absorption tests (Chapter 2, Section 2.5.5), which may be why very few *Candida* cells penetrated the material.

The artificial throat assay also supported the results of the *Candida* adherence assays by demonstrating reduced yeast biofilm on the surface-treated voice box prostheses (T1, T2 and T5). The artificial throat model has previously been found to produce a morphologically similar biofilm to that occurring on prostheses *in vivo* (Elving *et al.*, 2003). This was achieved by implementing a starvation cycle and exposing the prostheses to ambient air during the course of the assay (Elving *et al.*, 2003). It is important to note however, that saliva was not incorporated in the artificial throat model. In addition to this, the Groningen button at the front of the throat model was more heavily coated in biofilm than the button at the back of the model indicating intra-model variation in biofilm formation depending on the location of the button within the model.

Overall, three of the surface treatments (T2, T4 and T5) have been shown to have reduced Candidal adherence both in the presence and absence of saliva. This research has highlighted the differences between strains and species in relation to adherence and has demonstrated the extensive number of variables that are present when studying microbial adherence.

Conclusions

- Candida adherence was reduced significantly on surface-treated silicone rubber ($P<0.0001$).
- The reduction in *C. albicans* adherence did not appear to be the result of altered material surface hydrophobicity or functional group chain length.
- The presence of a salivary conditioning film increased *C. albicans* adherence to all the surface-treated materials but reduced the adherence of *C. tropicalis.*
- T2 (decrease not statistically significant), T4 and T5 materials reduced *C. albicans* adherence, when saliva was incorporated in comparison to the control.
- Both the penetration and artificial throat assays complemented and positively correlated with the results of the adherence assays.

Proposed adherence mechanisms:

Increased surface mobility due to the presence of siloxane bonds may be responsible for reduced adherence to naked surface-treated silicone rubber.

Two suggestions are put forward for the effect of saliva:

1. Saliva may be reducing the inhibitory effects of the underlying material to different extents depending on the amount of salivary constituents adsorbed. For example, T4 and T5, which reduced *Candida* adhesion may have adsorbed less saliva leaving more of their surfaces exposed.
2. The surface-treated material could have affected the adsorption of specific proteins into the conditioning film, reduced adsorption of PRPs, or enhanced adsorption of proteins inhibitory to *Candida* to T4 and T5 materials.

Chapter 4

Interaction of *Candida* with proteins and their influence on adherence to surface-treated silicone rubber

4.1 Introduction

A conditioning film is a coating comprised of proteins, electrolytes and other molecules which adsorb on to interfacing surfaces including tissues and biomaterials (Chapter 1, Section 1.5.3). The composition of a conditioning film is dependent on its location within the body. In the oropharyngeal cavity, the conditioning film is predominantly of salivary origin and consists mainly of salivary proteins (Everaert *et al.*, 1998a). A salivary pellicle develops rapidly on oral surfaces and certain proteins are believed to act as receptors for subsequent microbial adhesion (O'Sullivan *et al.*, 1997; 2000). Nikawa *et al.*, (1993) investigated the interaction between denture-soft lining materials coated with protein pellicles and *C. albicans*. In these investigations, the presence of saliva and serum resulted in a higher level of candidal attachment. The effect of a conditioning film is however complex and other research has suggested that the salivary pellicle inhibits microbial attachment (Busscher *et al.*, 1997; Jones *et al.*, 2001; McCourtie *et al.*, 1986; Chapter 1, Section 1.5.3). For example, salivary histatins have antifungal properties and possess a specific candidacidal domain (Bercier *et al.*, 1999; Raj *et al.*, 1990; Tsai and Bobek, 1998), which is clearly a protective component of saliva acting against the adherence of *Candida*. In contrast, proline-rich proteins (PRPs), have been identified as receptor molecules for *C. albicans* adherence (O'Sullivan *et al.*, 1997).

4.1.1 Proline-rich proteins (PRPs)

PRPs are synthesised in the acinar cells of the parotid glands, located within a well defined capsule in the cheek. There are three subsets of PRPs (Chapter 1, Section 1.5.4.1) ranging in molecular weight from 6 to 36 kDa. The PRPs are believed to have several roles within the oral cavity. They are present in the dental pellicle (Kousvelari *et al.,* 1980), and since they have a high affinity for calcium ions are believed to prevent the unwanted deposition of calcium on the tooth surface (Moreno *et al.,* 1979). PRPs also bind avidly to tannins, which are components of plant food, and large amounts of ingested tannins are associated with carcinomas. PRPs may therefore be the first line of defence against dietary tannins (Mehansho *et al.,* 1987). PRPs also play an important role in mediating bacterial adherence to oral surfaces. This is evident with *Streptococcus* and *Actinomyces* species (Gibbons, 1989; Hsu *et al.,* 1994; Stromberg *et al.,* 1996; Hallberg *et al.,* 1998), *Porphyromonas gingivalis* (Gibbons, 1989) and *Fusobacterium nucleatum* (Gillece-Castro *et al.,* 1991). O'Sullivan *et al.*, (2000) also demonstrated the importance of basic PRPs in the adherence of *C. albicans*. In the study, PRPs were isolated from human parotid saliva by gel filtration, and then separated further using electrophoresis. The PRPs were then transferred to nitrocellulose membranes, which were incubated with [^{35}S] methionine-radiolabelled *C. albicans* forming a cell overlay adherence assay. An autoradiogram of the membrane detected *Candida* binding to the PRP bands. Five strains of *C. albicans* adhered to a subset of four proteins with apparent molecular masses of 17, 20, 24 and 27 kDa. The N-terminal amino acid sequence of the bands indicated they were basic PRPs.

The results from Chapter 3 demonstrated that *C. albicans* adherence was reduced on several of the surface-treated silicone rubber samples (T2- reduction not significant, T4 and T5) when pre-coated with saliva for 18 h. This reduced adherence did not appear to be related to the surface hydrophobicity of the material and therefore other factors required investigation. As suggested in Chapter 3, it is possible that a salivary conditioning film binding to the surface-treated material, was influencing subsequent microbial adherence. Since salivary PRPs are the likely candidate proteins involved in mediating candidal adherence, the ability of these proteins to adsorb to the new materials becomes significant.

4.1.2 Fibronectin (FN); a protein that binds *C. albicans*

Biomaterials implanted in tissue will interface with the extracellular matrix (ECM) Fibronectin (FN) proteins are a major component of host ECM (Yan *et al.,* 1996) and are also present in fluids such as plasma. Fibronectins are high molecular weight glycoproteins which exist as dimers. The dimer is comprised of two non-identical subunits covalently linked near the COOH-termini by a pair of disulphide bonds. Around 20 isoforms of FN exist, some soluble and others non-soluble. The non-soluble forms occurs in the ECM and other matrices and in this form, the dimer associates into disulphide-bonded oligomers and fibrils. Soluble forms of FN are present in body fluids and are predominantly dimeric. The *C. albicans* cell wall contains multiple FN-binding proteins (Glee *et al.,* 1996; Gozalbo *et al.,* 1998), and FN may play an important role in the initiation and dissemination of *C. albicans* infections (Yan *et al.,* 1996).

4.2 Objectives

- Isolate and characterise PRPs from mixed human parotid saliva.
- Confirm the role of PRPs and fibronectin as adhesin receptors for *C. albicans.*
- Assess the effect of PRPs and fibronectin adsorption on surface-treated silicone rubber and subsequent candidal adherence.

4.3 Materials and methods

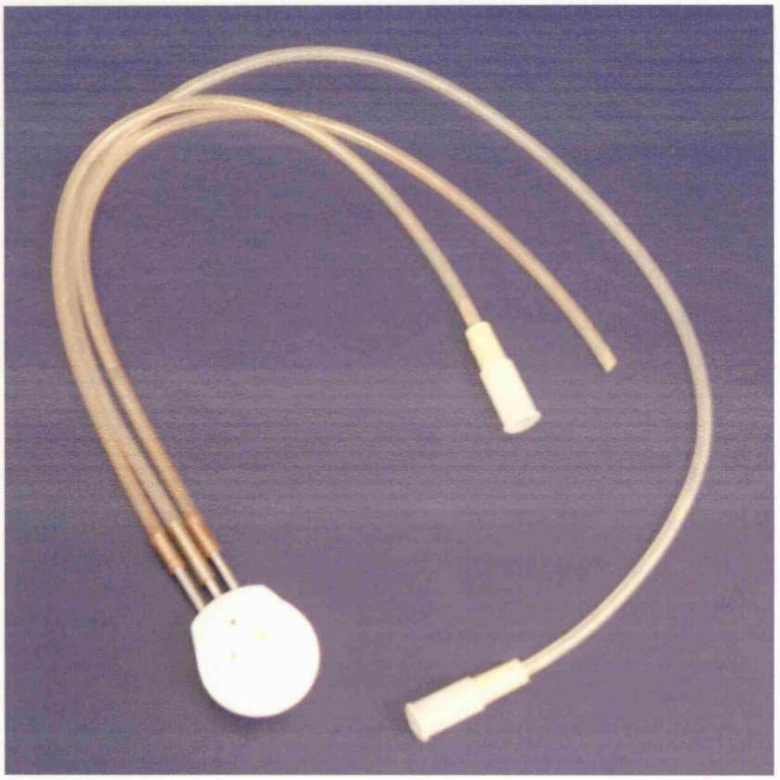
4.3.1 Parotid saliva collection

Parotid saliva was collected using modified Carlson-Crittenden Cups (Shannon *et al.*, 1962; Figure 4.1). Stimulated saliva was obtained from 10 volunteers (5 male, 5 female; mean age of 31 ± 10) on separate occasions. Saliva stimulation was through the use of 1% citric acid (1 ml; Cardiff Dental Hospital Pharmacy, Cardiff, UK) which was deposited on to the tongue. Approximately 2 ml of saliva was collected into ice-chilled universal containers from each volunteer.

4.3.2 Parotid saliva protein separation

Mixed parotid saliva was clarified by centrifugation (Sorval Discovery 100 SE Hitachi, Connecticut, USA) at 12,000 g for 15 min at 4°C. Collected saliva was either frozen at -18°C until required, or equal volumes added to Tris/HCl buffer (20mM Tris/NaCl, pH 8.0; 0.5M NaCl) and passed through a 0.22 μm filter (Millex, Cork, Ireland). A 5-ml volume was then loaded on to a sephacryl S200 gel filtration column (Amersham Biosciences, Amersham, UK), which had a fractionation range of 5-250 kDa. The column was connected to an Äkta fast performance liquid chromatograpghy (FPLC) system (Amersham Pharmacia Biotech, Uppsala, Sweden) and prior to use, was calibrated using a 1:1 mixture of para-nitrophenol (Sigma) and dextran blue (Sigma) and

Figure 4.1: Modified Carlsson-Crittenden cup used for the collection of parotid saliva. The white 'cup' was placed over the parotid glands and saliva was collected via one of the tubes with the aid of a suction pump whch was attached to the other tube.



four known standard proteins (β-amylase, alcohol dehydrogenase, albumin and carbonic anhydrase; Sigma). The elution times of these proteins were used to standardise the column. The column was equilibrated with 200ml of 0.5 M NaCl in 20 mM Tris/NaCl buffer (degassed and filtered) and eluted at a rate of 3 ml/min. Protein fractions were monitored by measuring the absorbance at 280 nm. Absorbance peaks identifying the protein fractions were measured by the Äkta Software (Amersham Pharmacia Biotech) and the eluents corresponding to equivalent peaks were pooled providing the final protein fractions. The partition coefficents (K_{av}) of the relevant protein peaks were calculated using the column void volume (Vo), peak elution volumes (Ve) and total column volume (Vt), as determined by the elution profile.

The protein fractions were dialysed for 5 days at 4°C in visking tubing (pore size; 5 kDa Scientific Instrument Centre, Eastleigh, UK) against distilled water containing protease inhibitors (5 mM n-ethylmaleimide, 1 mM iodoacetic acid, 5 mM benzamidine hydrochloride; Sigma). Fresh distilled water and protease inhibitors were added every 18 h. The protein fractions were then lyophilised and reconstituted in sample buffer (4.3.3.1) prior to use.

4.3.3 Saliva characterisation

4.3.3.1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular size of the protein fractions was determined by Phast System polyacrylamide electrophoresis (Pharmacia LKB, Basingstoke, UK). Lyophilised

samples were reconstituted in 10 µl of sample buffer (0.062 M Tris HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate, 5% 2-β-mercaptoethanol, 0.002% bromophenol blue) and then heated at 100°C for 5 min. A molecular weight marker (Sigma; Table 4.1) was similarly prepared to a concentration of 3 mg/ml.

Protein fractions were separated using 10-15% gradient polyacrylamide gels (PhastGel, Amersham Biosciences, Uppsala, Sweden). The gels were placed on the separation bed and buffer strips (Amersham Pharmacia Biotech) positioned into the anode and cathode compartments. Samples and standards were applied to the gel using a six lane, 1 µl application comb. Electrophoresis was performed at 15°C and involved two steps; an initial low electric output (100 V, 1.0 MA, 1.0 W, 4.0 Vh), which drew samples into and through the stacking gel, followed by an increased electrical output (250 V, 10.0 MA, 3W. 66 Vh) for separation of molecular components. Following electrophoresis, the protein fractions were stained with a PhastGel silver stain kit (Amersham Biosciences) in accordance with the manufacturer's recommendations. Molecular weights of the bands were calculated using R_f (rate of flow) values.

4.3.3.2 Amino acid analysis

Protein fractions (1 mg) were dissolved in 100 µl of deionised and distilled water and 20-µl of the preparation added to a glass hydrolysis tube. A further 100 µl of deionised and distilled water with 120 µl of HPLC-grade concentrated HCl (BDH-Aristar; Poole, UK) was then added. The hydrolysis tubes were sealed under vacuum and hydrolysed at

Table 4.1: Table of molecular weight marker proteins used for SDS-PAGE analysis.

Protein	Molecular weight (Da)
α_2-Macroglobulin	180,000
β-Galactosidase	116,000
Phosphorylase b	97,400
Serum albumin	66,000
Fumarase	48,500
Carbonic anhydrase	29,000
β-Lactoglobulin	18,400
α-Lactalbumin	14,200
Aprotinin	6,500

108°C for 18 h. The hydrosylates were subsequently desiccated with fused calcium chloride (Fisher Scientific) and phosphorus pentoxide (Sigma) under vacuum for 3 days. Samples were then washed with 1 ml of deionised and distilled water and desiccated twice to remove excess acid. After drying, the samples were dissolved in 50 µl of deionised and distilled water and filtered through 0.22 µm filter tips (Millex, Bedford, USA). Samples (20 µl) were then injected into a cation exchange amino acid column (Pickering Laboratories, Camberley, UK) interfaced with an HPLC system (Dionex UK Limited). In this system, the amino acids were added to an acid solution (pH 3.15; Pickering Laboratories), producing cations that were absorbed by the cation exchange resin. Sodium buffer (Dionex UK Limited, Camberley, UK) was used to elute the column, initially at pH 3.15, followed by a series of buffers (pH 7.4 and pH 11.0). Individual amino acids in the post-column eluent were then detected by reaction with Trione® ninhydrin reagent (Pickering Laboratories) at 55°C and monitored by absorbance at 570 nm. The peak heights and retention times were calculated using Dionex A1-450 chromatography automation software (Dionex UK Ltd). The amino acids were quantified by comparison with a standard amino acid solution (Sigma) applied to the column at known concentration.

4.3.4 Determining the interaction of *Candida* with proteins using a BIAcore™ 3000

The binding of *Candida* to PRPs and fibronectin (FN; soluble bovine; Sigma) was measured using a BIAcore™ 3000 biosensor (BIAcore AB, Uppsala, Sweden; Figures 4.2 and 4.3). CM 3 Sensor chips (BIAcore AB) were selected with short dextran matrices since these are designed for large analytes such as cells. Since each sensor chip

Figure 4.2: BIAcore 3000 biosensor

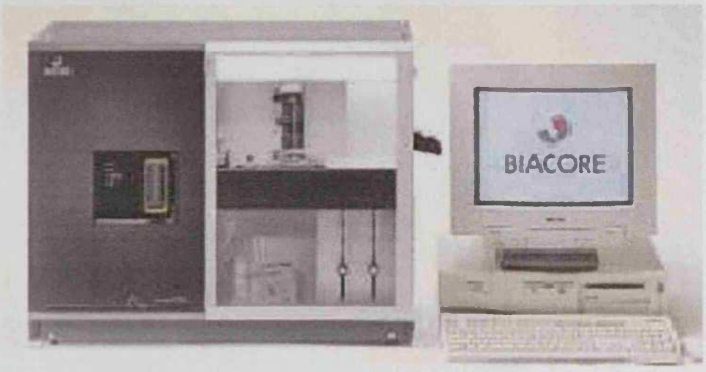
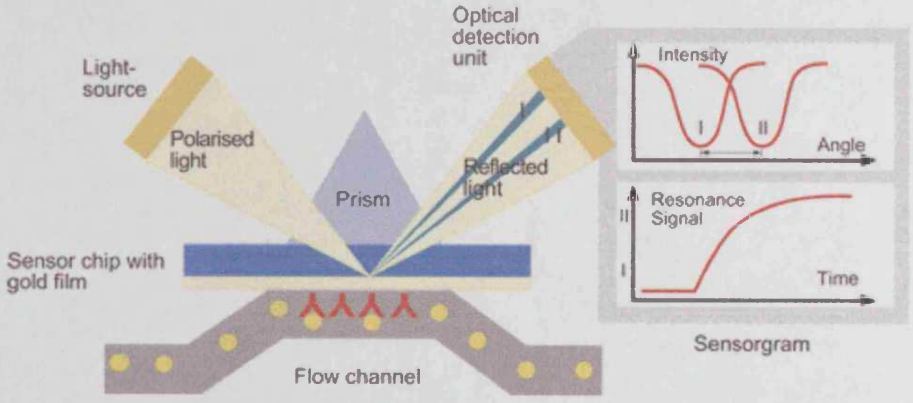


Figure 4.3: Schematic representation of surface plasmon resonance. As binding levels change, the angle of polarised light is altered and is detected by the optical detection unit.



housed 4 flow cells (FCs), FC 1 was selected as a control surface (no protein bound) and FC 2 provided the test surface with bound protein (ligand). Similarly, FC 3 provided the control surface for FC 4 in additional experiments. In this manner, a single sensor chip would allow experimentation with 2 separate protein ligands covalently coupled to FC 2 and FC 4. During ligand immobilisation, the control FCs were treated in the same manner as the test FC, except no ligand was injected.

4.3.4.1 Immobilisation of ligand on a CM3 sensor chip

pH scouting

The approach chosen to immobilise the ligands on the surface of the CM3 chip was by amine coupling using reactive esters. Preliminary experiments involved identifying the appropriate pH buffer that would allow electrostatic interaction of the ligand (immobilised protein) with the matrix of the CM3 chip. Ligands were diluted to 100 μg/ml in a range of sodium acetate pH buffers (10 mM; pH 4.0, 4.5 and 5.0). The proteins were injected over the sensor surface and the interaction determined based on the change in response units (RUs).

Amine coupling

All reagents used for amine coupling were supplied by BIAcore (amine coupling kit). The carboxymethyl groups on the matrix surface of a CM3 sensor chip were chemically

activated by injecting (continuous flow rate 5 µl/min HEPES buffer) a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N'- (dimethyl-aminopropyl)-carbodimide (EDC) to generate active ester groups. A 35-µl volume of NHS/EDC mix was then injected (7 min injection) followed by the protein ligand (FC 2 or FC 4) in the appropriate pH buffer. A targeted immobilisation level of 2000 RU was pre-selected using the BIAcore Wizard Software. Consequently, the volume of ligand injected was pre-determined by the BIAcore.

Deactivation

Deactivation of excess reactive groups on the surface involved a 7 min pulse injection of 1 M ethanolamine hydrochloride, pH 8.5. The high ionic strength of this solution removed non-covalently bound material from the surface.

Analyte binding to the immobilised ligand

In these experiments, the analyte was whole cells of *Candida*. The selected species and strains were *C. albicans* GDH 2346, *C. tropicalis* MMU1 (clinical isolate) and *C. krusei* NCYC 993 (non-clinical isolate) and these were cultured separately in YNB supplemented with 500 mM sucrose (Chapter 3, Section 3.3.2). After harvesting and washing in HBS-EP buffer (BIAcore) the yeast were adjusted to a concentration of 1×10^7cells/ml. The C*andida* suspensions were then separately injected over both the control and test FCs at a rate of 20µl/min for 30 s. After each injection, regeneration of

the sensor surface was achieved by an injection (20 µl/min for 30 s) of 50 mM glycine NaOH (BIAcore) to remove the bound *Candida.* All the *Candida* isolates were tested on at least two occasions and the highest level of binding was recorded.

4.3.5 *Candida* adherence assay to surface-treated silicone rubber pre-coated with Proline-rich proteins or Fibronectin

4.3.5.1 Surface-treated materials

Materials surface treated with (Tridecafluoro-1,1,2,2-tetrahydrooctyl)triethoxysilane (T2), acetoxytrichlorosilane (T4) and n-octadecyltrichlorosilane (T5) were assessed for their interaction with *Candida* after pre-coating with PRPs or fibronectin.

Candida albicans GDH 2346 was the species selected for use in these assays. Silicone rubber squares (4×2×1 mm; three duplicates of each surface treatment and three untreated controls) were immersed in a 2 mg/ml solution of either FN (derived from bovine plasma, Sigma) or PRPs (prepared from above) in distilled water for 18 h at room temperature. On removal, the squares were gently rinsed in distilled water to remove excess protein. The *Candida* adherence assay was then performed (Chapter 3, Section 3.3.2) on the protein-coated samples. Controls included untreated silicone material with and without the protein pre-coating and treated silicone rubber without protein pre-coating. The assays were repeated on two separate occasions and the data ware analysed according to Chapter 3, Section 3.3.2.

4.4 Results

4.4.1 Gel filtration of parotid saliva

A number of small protein peaks were detected by gel filtration chromatography at the commencement of the run followed by three larger peaks at the end. These larger peaks had K_{av} values of 0.24, 0.42 and 0.60 (Figure 4.4) and correlated to proteins of molecular sizes between 22.4 and 66 kDa.

4.4.2 Saliva characterisation

4.4.2.1 SDS-PAGE

SDS-PAGE revealed clear protein bands after silver staining (Figure 4.5). The protein fractions with K_{av} values of 0.42 and 0.60 showed two distinct bands of slightly different size but both were situated between the ß-lactoglobulin (18.4 kDa) and carbonic anhydrase (29 kDa) bands on the molecular weight marker. Another faint band at approximately 66 kDa was also visible on the 0.42 K_{av} band. This molecular weight does not correspond to proline-rich proteins and was not analysed further. It could have been contamination or another salivary protein. The R_f value for the most prominant band corresponding to the fraction with a K_{av} of 0.42 was 4.46 and other 0.83. Using the R_f values of the markers, the molecular weights of these proteins were calculated as 29 and 22.4 kDa respectively.

Figure 4.4: Elution profile of human parotid saliva from a sephacryl S200 gel filtration column over a period of 140 min. Peaks represent protein fractions which were separated on the basis of molecular size. Labelled are the two peaks which contained proline-rich proteins and V_o (Void volume) and V_t (total volume of column). Also displayed are the points at which the molecular weight markers eluted from the column although they were not loaded onto the column for this particular elution profile.

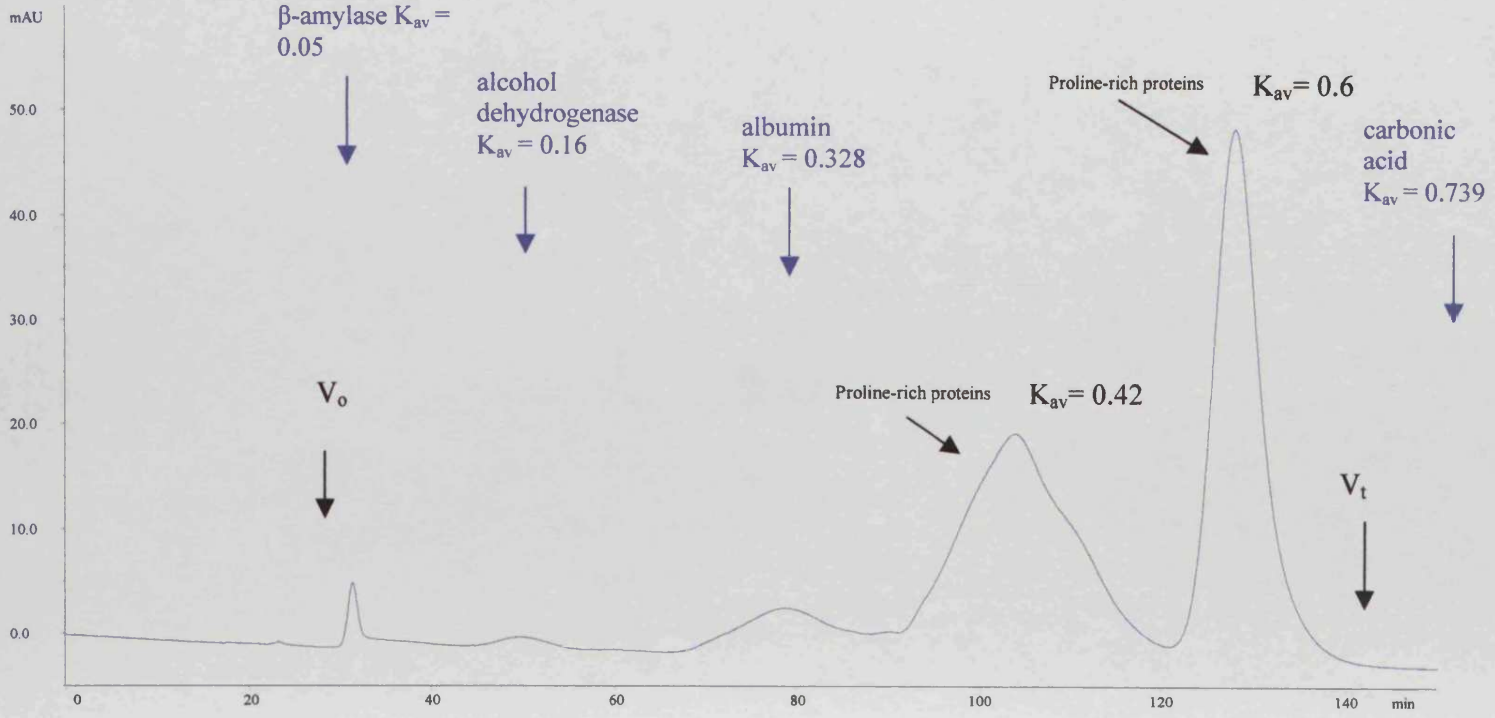
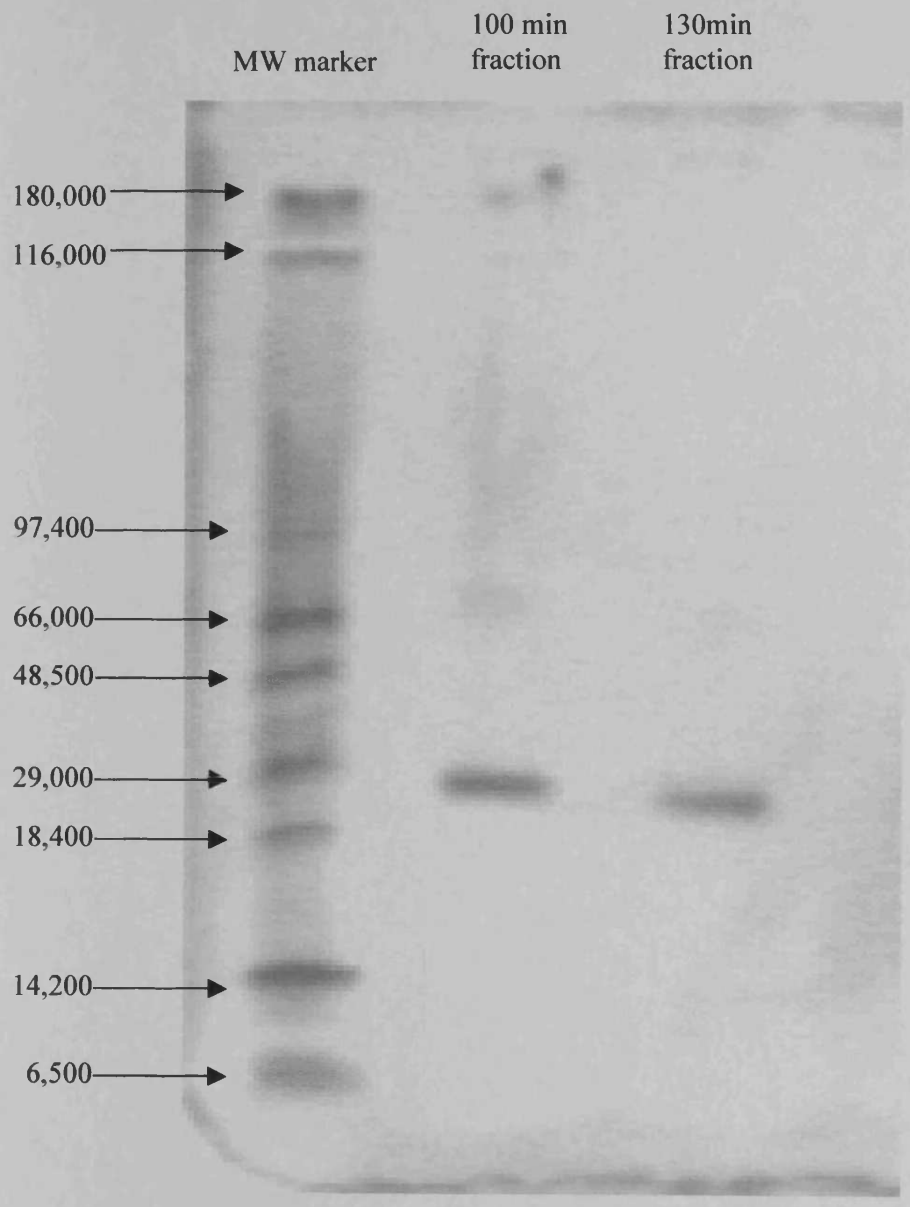


Figure 4.5: SDS-PAGE gel displaying protein fraction separated on the basis of molecular size



4.4.2.2 Amino acid analysis

Amino acid analysis on the two fractions of molecular sizes 29 and 22.4 kDa revealed that they were composed of high proportions of glutamine; 289 amino acid residues/1000, proline; 201 amino acid residues/1000 and glycine; (250 amino acid residues/1000. These three amino acids constituted 74% of the total protein structure for both fractions. The protein fractions also contained small amounts of aspartate, serine, alanine, valine, isoleucine, leucine, lysine, histidine and arginine (Figure 4.6).

4.4.3 Determining the interaction of *Candida* with proteins using the BIAcore™ 3000

Fibronectin and PRPs were successfully immobilised on to the BIAcore sensor chip (Figures 4.7 and 4.8). The target number of RUs of each protein to be immobilised on the chip was 2000 (~2 ng/mm^2). The actual amount of fibronectin bound was 1801.4 RU and the amount of PRPs was 1993.9 RU. *Candida albicans* GDH 2346 bound to both fibronectin (30.6) RU (Figure 4.9) and PRPs (154 RU; Figure 4.10). No binding was detected with *C. tropicalis* or *C. krusei*.

4.4.4 *Candida albicans* adherence assay to surface-treated silicone rubber pre-coated in proline-rich proteins or fibronectin

The adherence of *C. albicans* to control silicone rubber pre-coated with FN was significantly higher than adherence to uncoated samples confirming previous findings (Section 4.4.3). However, increases in adherence to treated samples were only statistically significant for T2 ($P<0.02$) and T4 materials ($P<0.04$; Table 4.2). The adherence of *Candida* to samples pre-coated with PRPs was inconsistent, with large differences evident between fields of view. One way analysis of variance (ANOVA) demonstrated no statistically significant difference between the adherence of *Candida* to the PRP-coated and control samples.

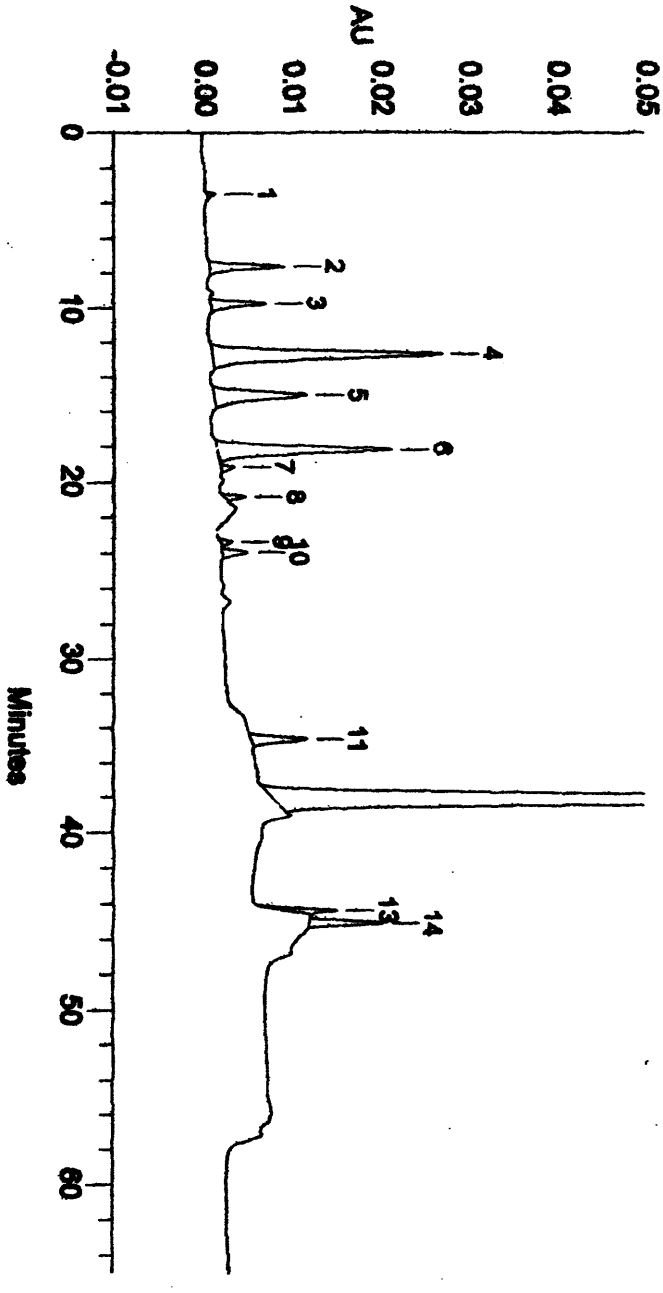


Figure 4.6: HPLC elution profile of amino acids contained within PRP fractions. Peak 4 – glutamine, Peak 5 – Proline, Peak 6 – glycine.

Figure 4.7: BIAcore 3000 immobilisation of FN to a BIAcore CM3 sensor chip.

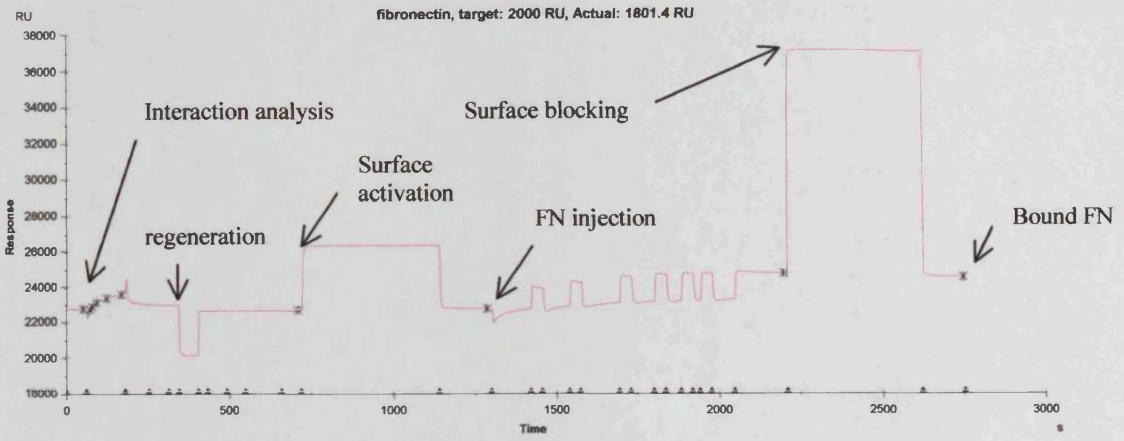


Figure 4.8: BIAcore 3000 immobilisation of proline-rich proteins onto a BIAcore CM3 sensor chip.

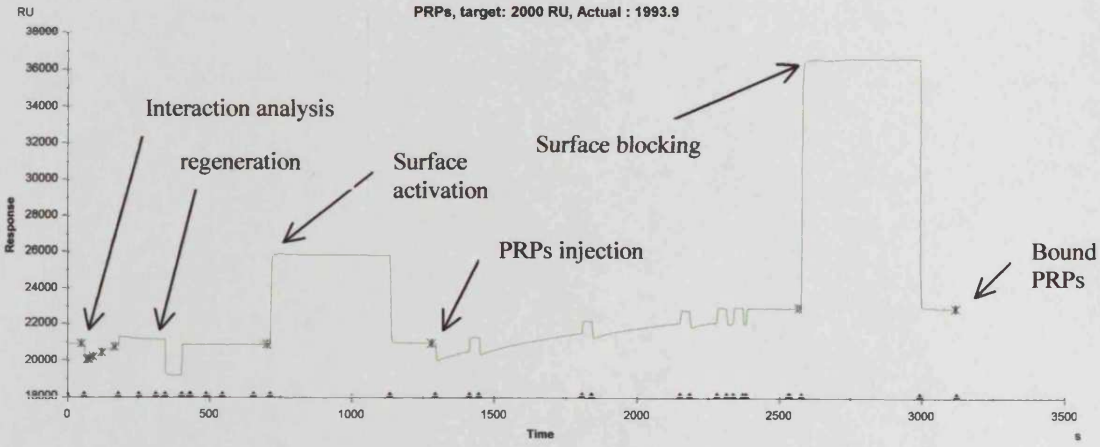


Figure 4.9: BIAcore sensorgram displaying the binding of *C. albicans* GDH 2346 onto a FN-immobilised sensor chip.

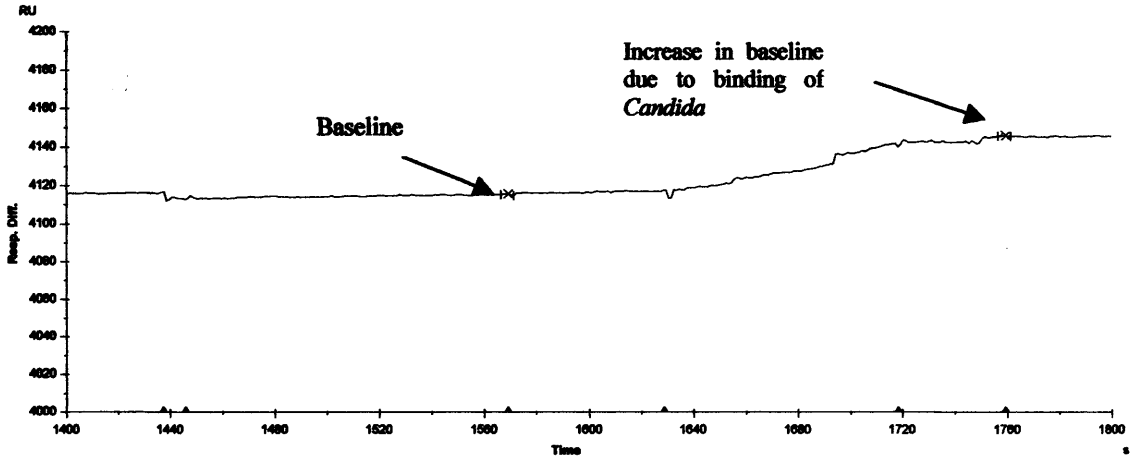


Figure 4.10: BIAcore sensorgram displaying the binding of *C. albicans* GDH 2346 to a proline-rich protein-immobilised sensor chip.

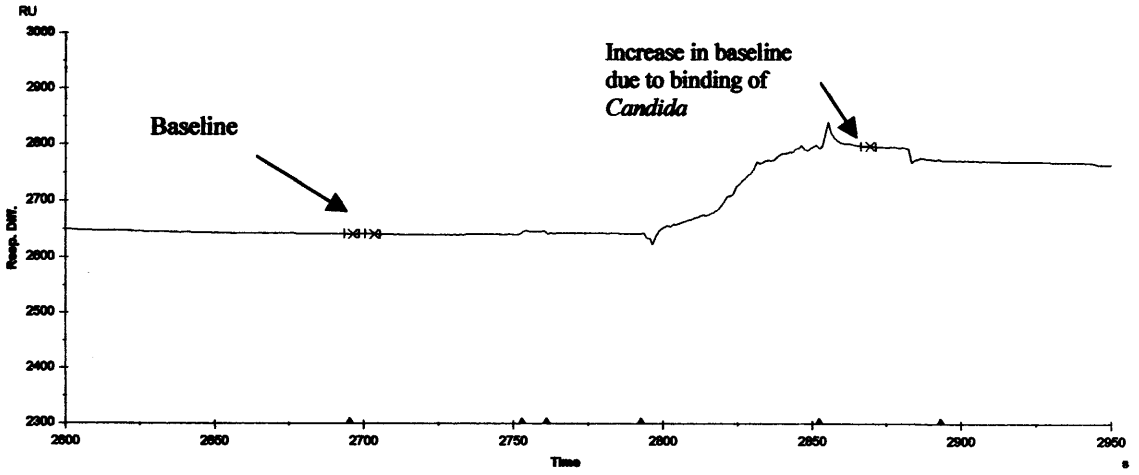


Table 4 2: Adherence/mm^2 of *C. albicans* GDH 2346 to surface-treated silicone rubber coated with PRPs and FN.

Surface treatment	Adherence of *C. albicans* GDH 2346/mm^2		
	No protein coating	PRPs	FN
Control	1184 ± 706	1030 ± 1169 *ns*	2499 ± 1306 *ns*
T2	184 ± 219	4525 ± 4619 *ns*	1015 ± 447*
T4	259 ± 181	296 ± 103 *ns*	3565 ± 2486*
T5	11 ± 11	720 ± 389 *ns*	2488 ± 2197 *ns*

* *P<0.05*
ns- not significant

4.5 Discussion

4.5.1 Protein separation and characterisation

The human PRPs represent a heterogeneous group of proteins that comprise about 70% of the parotid proteins (Schenkels *et al.*, 1995). The PRPs are characterised by a predominance of the amino acids proline, glycine and glutamine which make up 80% of all PRP amino acids. PRPs are classified into three groups: acidic, basic and glycosylated. The acidic and basic proteins are relatively small ranging in molecular weight from 6-27 kDa and the glycosylated proteins are slightly larger at about 36 kDa. O'Sullivan *et al.*, (1997), demonstrated that *C. albicans* bound to a subset of four proteins with molecular masses of 17, 20, 24 and 27 kDa. N-terminal amino acid sequencing of these 4 proteins revealed they were members of the basic PRP (bPRP) family. This current study utilised gel filtration chromatography to produce a number of protein fractions, which were separated by their molecular mass. The technique is ideal for separating PRPs due to their distinct molecular masses compared to many of the other salivary proteins. Other studies have also used this method for separating PRPs from parotid saliva (Carlén *et al.*, 1998; O'Sullivan *et al.*, 1997; Strömberg *et al.*, 1992).

The protein fractions eluted from the column earlier than anticipated indicating they were of a larger molecular mass than expected. However, most PRPs exist as dimers (Yao *et al.*, 2004), which may have accounted for this early elution. Such protein dimers would have been denatured in to single proteins prior to electrophoresis by the 2-β-

mercaptoethanol within the SDS-PAGE loading buffer which degrades the disulphide bonds joining the proteins. SDS-PAGE revealed that two fractions contained proteins of molecular sizes of between 29 and 22.4 kDa (the same size as the basic and acidic PRPs, which are between 6 and 27 kDa according to O'Sullivan *et al.*, 1997 and 11-31 kDa according to Schlesinger *et al.*, 1994). Amino acid analysis was used to characterise these two fractions since PRPs have a distinct make-up of 80% proline, glycine and glutamine meaning easy identification using this method. Amino acid analysis revealed that 74% of the protein comprised of these amino acids indicating a high proportion of PRPs in these fractions. Alternative methods for the identification of proteins include Western blotting where a labelled antibody specifically binds to an antigen of the target protein. Whilst Western blotting is an accurate means of detecting proteins, there are currently no commercially available antibodies against PRPs. Protein sequencing is another technique utilised in the identification of proteins and was the method adopted by O'Sullivan *at al.*, (1997). However, protein sequencing was not an available option during this study.

4.5.2 *Candida* binding assays

Fibronectin (FN) was employed in both the BIAcore and adherence assays because it is known to interact with *C. albicans* (Glee *et al.*, 1996; Gozalbo *et al.*, 1998; Yan *et al.*, 1996) and is considered a potential mediator of *C. albicans* adherence. It was therefore of importance to investigate its adsorption to the novel silicone rubber surfaces and assess the effect it had on *C. albicans* adherence to the new materials. Furthermore

since the FN was purchased commercially and its purity assured it was also an ideal control molecule for experimental design for subsequent PRPs investigations.

The BIAcore™ (Biomolecular Interaction Analysis) biosensor allows real-time measurement of the binding of two macromolecules. One macromolecule (ligand) is immobilised on to a sensor chip and the second macromolecule (analyte) is injected over the surface of the sensor chip. Binding of the analyte to the ligand is measured via a change in surface plasmon resonance (SPR) signal. This is an optical phenomenon arising in thin metal films under conditions of total internal reflection. The phenomenon produces a sharp dip in the intensity of reflected light at a specific angle known as the resonance angle. Changes in the mass concentration of macromolecules at the sensor interface changes the resonance angle. Hence, as the analyte binds to the immobilised ligand, the resonance angle changes and a response is registered. The response is expressed in resonance units (RU) and displayed on a sensorgram. A response of 1000 RU corresponds to a shift of 0.1° in the resonance angle. This in turn represents a change in surface protein concentration of about 1 ng/mm^2. Recent studies have used the BIAcore system to demonstrate the binding of *Streptococcus* species to FN (Kawashima *et al.*, 2003).

In this present study, both FN and PRPs were shown to bind to *C. albicans* as previously reported (O'Sullivan *et al.*, 1997; Yan *et al.*, 1996). No interactions between PRPs and *C. krusei* or *C. tropicalis* were evident which may further support the binding

specificity of *C. albicans* to PRPs and may explain why these species are less frequently encountered within the oral cavity.

The binding of *C. albicans* to FN indicates its potential importance as a mediator in pathogenic processes. Possible mechanisms for FN binding to *C. albicans* remain undetermined but a cell-wall associated form of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is believed to be involved (Gozalbo *et al.*, 1998) along with cell wall proteins that are homologs of mammalian integrins (Santoni *et al.*, 1995) and 60 and 105 kDa glycoproteins (Klotz *et al.*, 1994). In addition to this, many proteins in the ECM, including fibronectin contain the tripeptide amino acid sequence arginine-glycine-aspartic acid (RGD) and *C. albicans* cells are known to contain an adhesin capable of binding this amino acid sequence (Hostetter, 2000).

4.5.3 *Candida* adherence assay to surface-treated silicone rubber pre-coated with Proline-rich proteins or fibronectin

The objective of these experiments was to ascertain whether materials previously found to inhibit candidal adherence remained effective in the presence of proteins known to mediate adherence. It was therefore hypothesised that the adherence of *C. albicans* to untreated silicone rubber pre-coated with either FN or PRPs would be higher than with uncoated material (Section 4.1). It was also predicted that the adsorption of PRPs may have been lower to T2, T4 and T5 materials as these materials had previously demonstrated reduced *C. albicans* adherence in the presence of saliva (Chapter 3,

Section 3.5.2.1). It was also speculated that these materials may have produced a conformational change in the protein causing deactivation of the *C. albicans*-specific binding site. Conformational changes are known to occur in PRPs as described by Hay *et al,* (1986). Their work demonstrated that despite PRPs having a strong affinity for certain strains of *actinomyces viscosus* when adsorbed to tooth surfaces, when in solution, PRPs lost their affinity for the bacterium. They postulated that when PRPs bind to apatite a conformational change occurs revealing previously hidden receptors for microbial attachment.

The results of the adherence assay with material pre-coated with FN supported the hypothesis that protein adsorption to the silicone rubber would increase microbial attachment by showing a significant increase in candidal adherence to FN-coated control silicone rubber. The adherence of *Candida* to material pre-coated with PRPs however displayed no significant difference from the adherence of the yeast to uncoated material. Particularly large standard deviations appear to be a factor in these results (Table 4.2). Large differences were noted in the adherence of *Candida* on different areas of the same sample. Some areas were densely packed with adhering yeast cells whereas in other areas, adherence was sparse. This feature probably accounted for the large standard deviations with these experiments. This clustering phenomenon was observed to some extent on all samples tested throughout the study but was more evident on PRP-coated material. It would have been interesting to determine the extent of coverage of the PRPs on the samples, as the high variability within the adherence results suggest an uneven spread of the proteins. However, such experiments were not feasible due to limited

availability of PRP samples. An additional factor which may have affected the results was the purity of the isolated PRPs. SDS-PAGE and amino acid analysis confirmed their presence, but there may have also been salts present in the sample suggesting the actual concentration of PRPs may have been lower than expected. However, the concentration of PRPs in whole saliva is 0-180 μg/ml and in human parotid saliva, 230-1251 μg/ml (Scannapieco, 1994). These concentrations are low compared to those used in this assay which were deliberately high to detect any effects. The lack of a significant difference in adherence produced by the presence of PRPs, suggests that in this instance, they have not played a role in candidal adherence. It may therefore be the overall quantity of salivary constituents adsorbing to the material which may be affecting variations in adherence as suggested in Chapter 3. It may be worth pursuing the search for a salivary protein whose adhesion may be inhibited by the silicone rubber surface treatments.

Conclusions

- Protein fractions containing a high proportion of PRPs were isolated and characterised.
- Binding of *Candida albicans* GDH 2346 to PRPs and FN was confirmed.
- Equivalent interaction of *C. tropicalis* and *C. krusei* with PRPs was not evident.
- No significant difference was evident between the adherence of *C. albicans* GDH 2346 to silicone rubber pre-coated with PRPs and uncoated silicone rubber.

Chapter 5

Adherence and motility of *Proteus mirabilis* on surface-treated silicone rubber

5.1 Introduction

The location of a biomaterial within the body determines the type of colonising microorganism. The adherence of *Candida* to modified silicone rubbers was investigated in Chapter 3 and this was based on medical devices used in the oral cavity and throat. The work in this chapter examines the adherence of the bacterium *Proteus mirabilis* to the surface-treated silicone rubbers. *Proteus mirabilis* was selected for study as it is a frequent coloniser of silicone rubber urinary catheters and a principle cause of urinary tract infections (UTIs) (Morris and Stickler, 1998; Winters *et al.*, 1995).

5.1.1 Urinary catheters

The primary function of urinary catheters is to relieve urine retention caused by anatomical or neurophysiological blockage, and to facilitate the repair of the urethra post surgery. Urinary catheters are also vital in the management of long-term urinary incontinence in the elderly and patients with spinal cord injuries. These patients can be reliant on urinary catheters for several months or even years. Unfortunately, this high dependency and chronic usage of urinary catheters can lead to associated UTIs. Catheter-associated UTIs (CAUTIs) are frequently asymptomatic (Tambyah and Maki, 2000) although if untreated the infection can ascend to the kidneys leading to potentially serious complications such as pyelonephritis and septicaemia. CAUTIs occur following bacterial adherence and biofilm formation on the surface of the catheter's inner lumen. Since a catheter disrupts normal bladder function and urinary

flow, the presence of even a small number of bacteria can have a significant effect. In the case of urease-positive bacteria, such as *P. mirabilis* (Chapter 1, Section 1.6.3.1) the problem is further compounded, since encrustation within the catheter lumen can create an obstruction within the catheter. The presence of urease elevates the pH of the urine by the degradation of urea and the production of ammonia. As the urine pH increases, crystallisation of calcium and magnesium phosphates occurs (Getliffe, 1994; Kunin *et al.*, 1987) and the resulting struvite and apatite crystals cause catheter encrustation (Morris and Stickler, 1998; Winters *et al.*, 1995). Furthermore, *P. mirabilis* has a dimorphic nature allowing it to differentiate from a short rod to an elongated, highly flagellated swarmer cell, which has the ability to migrate along the catheter surface. This added virulence factor enables the bacterium to colonise the whole length of a catheter in a short space of time.

Efficient catheter management can help prevent CAUTIs. Simple strategies such as the adoption of aseptic insertion methods and the regular replacement of catheters will reduce the risk of bacterial contamination (Newman, 1998). However, since CAUTIs are often asymptomatic it is difficult for patients, particularly for those who are elderly and in care, to recognise the early onset of an infection. As a result, unless patients are regularly visited by a health professional, CAUTIs can progress undetected. Prophylactic antibiotic therapies have been administered to catheterised patients but this can have a deleterious effect on normal microflora and promote the development of antibiotic resistance and fungal overgrowth (Wilcox and Spencer, 1992; Zhanel *et al.*, 1990).

Attempts have been made to produce antimicrobial surface coatings for catheters with silver or hydrogel-coated catheters now being commercially available (Chapter 1, Section 1.8.1.1). The success of these catheters has however been limited. As well as altering the chemical properties of the catheter surface, coatings are likely to influence the surface roughness of the urinary catheter and as discussed previously, (Chapter 1, Section 1.5.2) this can have a significant influence on microbial retention to a surface, which may be a factor in the failure of catheter coatings.

5.2 Objectives

- To determine the level of adherence of *P. mirabilis* to surface-treated silicone rubber with and without the presence of urine.
- To examine the effect of surface-treated silicone rubber on the motility of *P. mirabilis*.
- To measure the surface roughness of a variety of commercially available urinary catheters and determine any correlation between their roughness and *P. mirabilis* motility.

5.3 Materials and methods

5.3.1 Parallel-plate flow chamber adherence assay

5.3.1.1 Preparation of glass plates for silicone rubber spin-coating

Lipsol liquid detergent (0.5 ml; L.I.P Equipment and Services Ltd) was applied to glass plates (75×50×2 mm with rounded edges) and rubbed with a soft cloth. The plates were then soaked in hot 10% lipsol detergent for 5 min, rinsed in hot water for 2 min and again in distilled water. The plates were then soaked in 1% (w/v) ammonium persulphate (BDH Chemicals) solution in concentrated sulphuric acid (Fisher Chemicals) prior to rinsing with copious amounts of deionised water and dried in air.

5.3.1.2 Preparation of silicone rubber for spin-coating onto glass plates

Polymer mix (1.39 g; Chapter 2, Table 2.2) was dissolved in 40.5 g of chloroform (Fisher Chemicals) and 0.2 g (1% w/w) of platinum catalyst (ABCR UK Ltd) was added. The solution was centrifuged at 15,000 g (Sigma 3K15, Philip Harris Scientific, Harz, Germany) for 15 min to remove debris.

5.3.1.3 Spin-coating silicone rubber onto glass plates

Silicone rubber solution (1.5 ml) was applied to the surface of a clean glass plate, attached to the flat-headed attachment of a vertically clamped drill (Figure 5.1). Application of the drill to the solution at 2000 rev/min for 25 s resulted in an even dispersion of the silicone polymer solution over the glass slide (Downer *et al.*, 2003). The glass slide was heated at 100°C for 1 h to cure and then placed in a vacuum desiccator for 12 h to remove any volatiles.

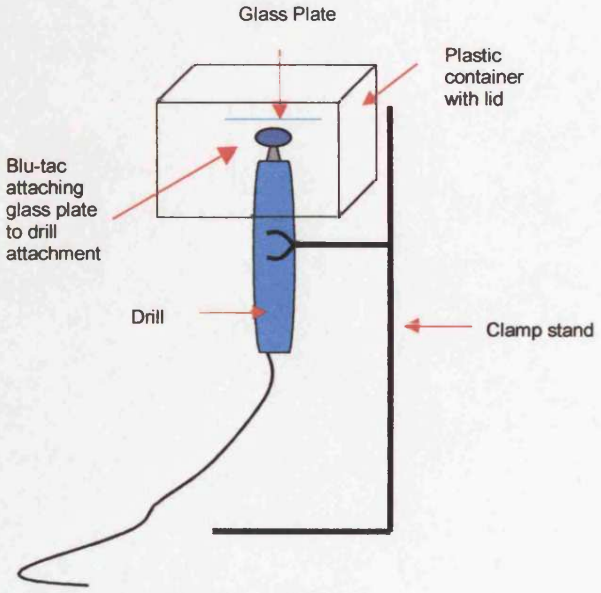
5.3.1.4 Confirmation of silicone rubber spin-coating

The glass plates were subjected to Fourier Transform Infrared Spectroscopy (FT-IR) using an Avatar 360 spectrometer (Thermo nicolet, Warwick, UK) to confirm the presence of silicone rubber on the glass slide. Thirty two scans of each material were taken and standardised against a background spectrum taken in air. A DTGS KBR detector was used which had a spectral range of 4000-675 cm^{-1}. FT-IR was also performed on silicone rubber as a comparison. The glass plates were examined under light microscopy (Olympus BX-40 phase contrast, London, UK) to assess material smoothness and the presence of any surface defects.

5.3.1.5 Silicone rubber surface modification

The silicone-coated glass slides were then surface treated using the method described in Chapter 2, Section 2.3.2. Silane treatments T1 – T6 (Chapter 2, Table 2.3) were used to treat the coated plates.

Figure 5.1: Schematic diagram of method used for spin-coating silicone rubber onto glass plates used in parallel-plate flow chamber.



5.3.1.6 Parallel-plate flow chamber adherence assay with *Proteus mirabilis*

I performed the parallel plate flow chamber assays at Cardiff University, Department of Biosciences. *Proteus mirabilis* (B2 laboratory strain, Cardiff University, UK; isolated from an infected urinary catheter) was cultured in tryptone soya broth (Oxoid) at 37°C for 16 h, under gentle agitation. The bacteria were centrifuged at 4000 g (Sorvall RC5C, Sorvall Instruments, Stevenage, UK) for 5 min and washed twice in distilled water. The resulting cell suspension was sonicated for 20 s to break aggregates and chains, and the bacteria were then enumerated using an Improved Neubauer Haemocytometer (Hawksley). The bacteria were adjusted to a concentration of 1×10^8 cells/ml in PBS for use in the flow chamber assay.

Glass plates and Teflon™ spacers from the parallel-plate flow chamber (approx 12 cm in length; Materia Technica, University of Groningen, Groningen, The Netherlands) were cleaned in 2% alkaline surfactant solution in deionised water (Decon 90; Decon Laboratories Ltd, Hove, UK). The plates and spacers were sonicated in the solution for 5 min, washed thoroughly in hot water for 2 min, rinsed in methanol and finally in deionised water.

Two glass plates were used in each flow chamber, (one untreated plate and one silicone rubber-coated plate). The silicone rubber-coated plate was positioned at the bottom of the flow cell with the silicone coating exposed to 'flow-through' bacterial suspensions. The assembled flow chamber was positioned on the stage of a microscope (Olympus BX-40 phase contrast microscope) equipped with an ultra long working distance objective (LC-ACH × 40PH, Olympus). The chamber was

connected to two reservoirs via silicone rubber tubes, one of which contained phosphate buffered saline (PBS; 0.01M, pH 7.3; Oxoid) and the other, a suspension of *P. mirabilis*. The solutions were injected through the chamber using a peristaltic pump (Watson-Marlow, Falmouth, UK; Figures 5.2 a and b). A valve system allowed the solutions to pass through the flow chamber without the introduction of an air-liquid interface, which could displace adhering bacteria from the plate surface. PBS was passed through the flow chamber for 10 min in order to remove air from the system and regulate flow rate. Prepared suspensions of *P. mirabilis* were then pumped continuously through the flow cell system for 5 h at a flow rate of 1.5 ml/min. All flow through experiments were performed at 37°C in a dedicated incubator room.

Direct observation of bacterial adherence over a 0.014mm^2 field of view was achieved using a video camera (CCD monochrome; VC600 Video Control Ltd) connected to the microscope with a camera mount adapter (Olympus, U-CMAD). A sequence of images (30 images/s) were captured using a monochrome frame grabber (Mach Series DT3155, Data Translation Ltd) and images of the same area were recorded every 10 min (for the first hour), every 30 min (next 2 h) and every 1 h (final 2 h). After 5 h, 6 images were randomly captured over the surface of the plate. A passage of air was then injected through the flow cell, followed by PBS and an image obtained to indicate bacterial displacement due to the air. Each silicone rubber surface treatment was tested on 3 separate occasions and adhered cells were counted manually. Unpaired t-tests were used to compare between two individual data sets and one-way analysis of variance (ANOVA) and Bonferroni post tests were used to compare between multiple data sets (GraphPad Prism).

Figure 5.2a: Schematic diagram of the parallel-plate flow chamber

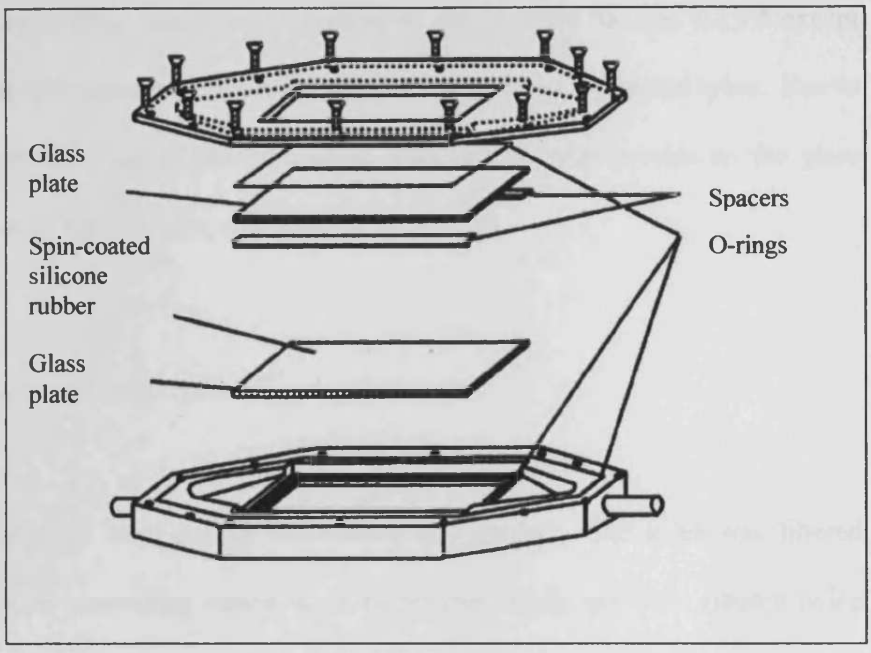
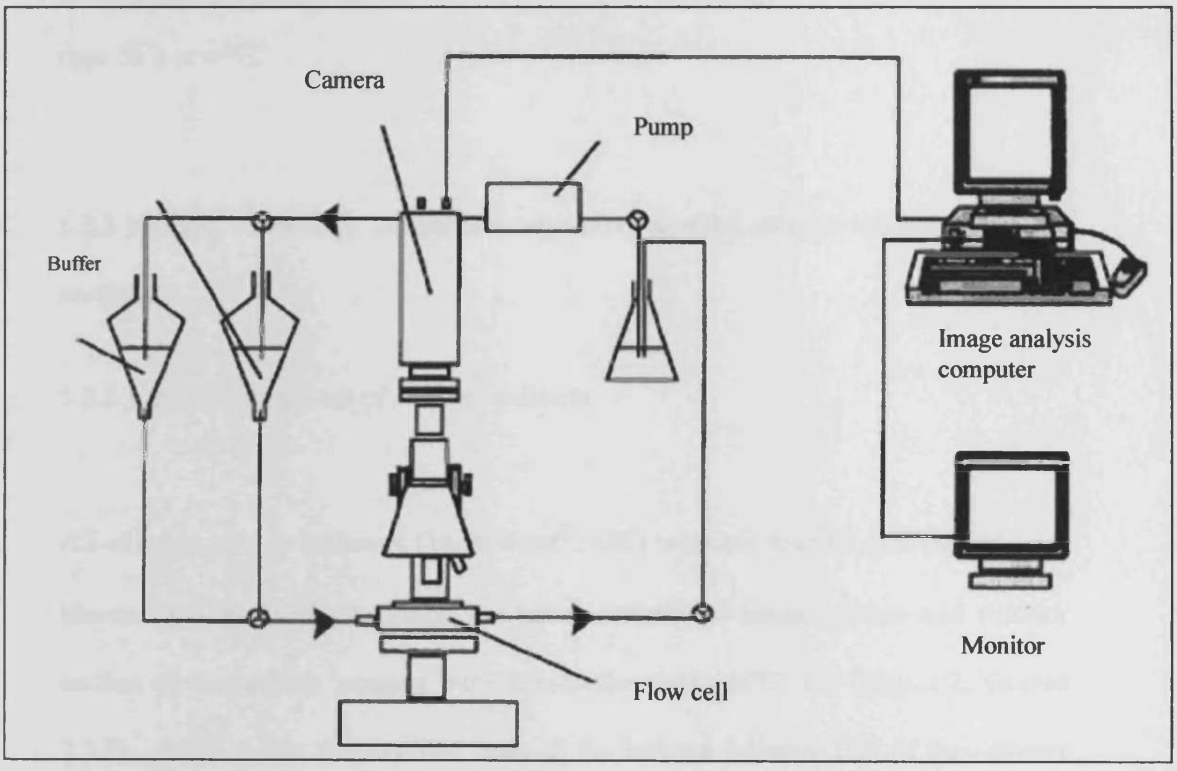


Figure 5.2b: Schematic diagram of the parallel-plate flow chamber system



5.3.1.7 Parallel-plate flow chamber assay incorporating urine

An assay incorporating urine was performed as described in Section 5.3.1.5 except after culturing and enumeration, the bacteria were suspended in human urine. Due to the crystallisation of urine, the percentage coverage of cells/crystals on the glass plate was used as a method for enumerating adherence.

Urine collection and preparation

Urine was gathered from 5 male individuals and pooled. The urine was filtered through a funnel containing cotton wool to remove debris and then filtered twice through a pre-sterilised capsule filter (0.2 μM; Sartorius, Epsom, UK) connected to a peristaltic pump (Watson-Marlow, Falmouth, UK). Urine was then stored for less than 36 h at 4 °C.

5.3.2 Motility of *Proteus mirabilis* over surface-treated silicone rubber catheter sections

5.3.2.1 Surface treatment of catheter sections

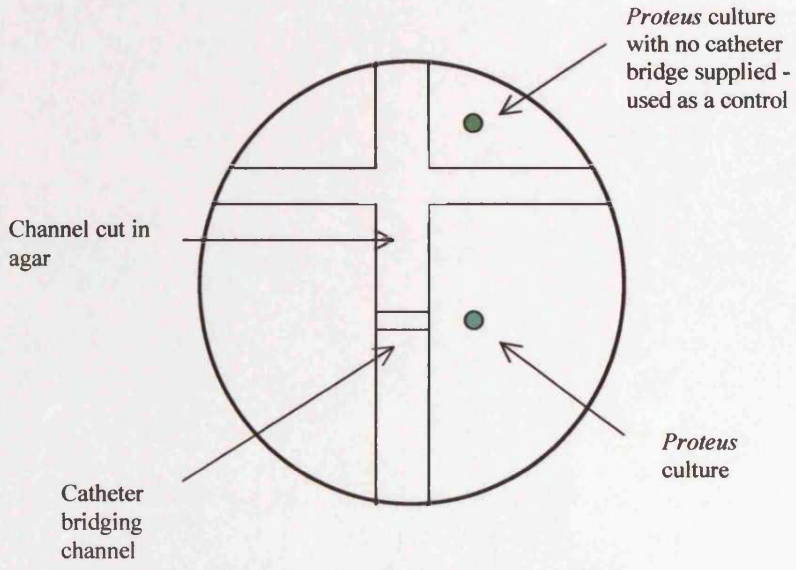
All-silicone urinary catheters (14ch; Bard®, UK) were cut into 10 mm sections and bisected longitudinally, exposing the lumen. Both the lumen surface and exterior surface of the catheter sections were then surface-treated (T1-T6; Chapter 2, Section 2.3.2). Prior to use, the exposed ends of the balloon inflation line of the catheter

sections were sealed with silicone medical adhesive (Silastic®, Dow Corning, Michigan, USA).

5.3.2.2 Migration of *Proteus mirabilis* over surface-treated catheter sections

The migration assay performed was adapted from Stickler and Hughes, (1999). *Proteus mirabilis* NSM42 (Laboratory strain; isolated from an infected urinary catheter) was cultured in tryptone soya broth (TSB; Oxoid, Basingstoke, UK) under gentle agitation for 4 h at 37 °C. Agar plates (90 mm diameter) containing 17 ml of tryptone soya agar (TSA; Oxoid) were prepared and dried at room temperature for 18 h. Channels were cut (8.5 mm) across the centre of each plate with an additional cut at right angles to the first channel. Plates were allowed to dry for an additional 3 h before aliquots (3×10 µl) of bacterial culture were inoculated 50 mm from the edge of the channel. The inocula were left to dry for 15 min before catheter sections were placed adjacent to two of the inocula, thus acting as a bridge across the channel (Figure 5.3). The sections were placed across the channel ensuring the catheter sections were not in contact with the base of the agar plate. As a control, the remaining inoculum was not provided with a catheter bridge. After incubating at 37°C for 18 h the plates were examined for migration of *P. mirabilis* across the catheter bridges on to the uninoculated sections of the plates (Figure 5.3). The frequency of migration across the catheter sections was used to assess the bacteria's ability to migrate across the catheter sections. Six replicates of each surface-treatment were tested on three separate occasions.

Figure 5.3: Schematic diagram of motility assay



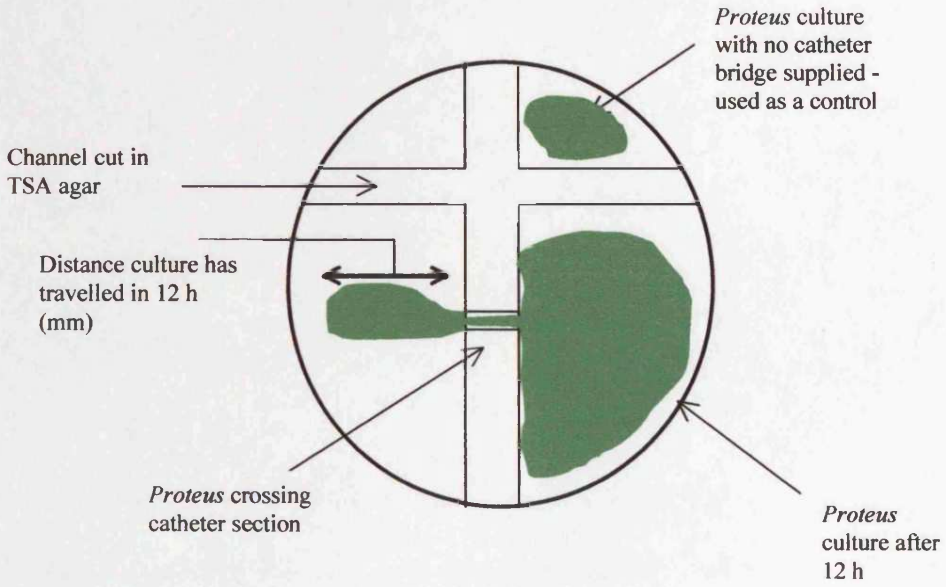
5.3.3 Motility assay on urinary catheters

Motility assays were performed on four types of commercially available urinary catheter; all silicone, hydrogel-coated latex, silicone elastomer-coated latex and silver/hydrogel-coated latex (Bard, Crawley, UK). The method used was as described in Section 5.3.2.2, except, the 10 mm catheter sections were not bisected. In addition the distance travelled by the colony from the edge of the channel on the uninoculated side of the plate to the far edge of the colony, was used as a measure of the rate of bacterial migration across the catheter. Six replicates of each surface-treatment were tested on three separate occasions (Figure 5.4).

5.3.4 Analysis of catheter surface roughness

Catheter surface roughness was assessed using AFM (Chapter 2, Section 2.3.5). Four types of catheters were tested: all-silicone, hydrogel-coated latex, silicone elastomer-coated latex and silver/hydrogel-coated latex (Bard). Small sections of these catheters were cut ensuring exposure of the lumens and eyehole edges. The sections were cut as small as possible to reduce their curvature as this can hinder AFM analysis. The sections were then adhered to microscope slides using silicone medical adhesive (Silastic®, Dow Corning) and images and roughness measurements were produced as described in Chapter 2, Section 2.3.5.

Figure 5.4: Schematic diagram of motility assay displaying the migration of *Proteus mirabilis* across the catheter section to the other side of the agar plate. Displayed is the measure of distance travelled by the culture after crossing the catheter section



5.4 Results

5.4.1 Silicone rubber spin-coating

The spin-coating of silicone rubber on to the glass plates was successful, as demonstrated by FT-IR spectra (Figures 5.5 and 5.6). The FT-IR spectra for the spin-coated glass plates displayed transmittance at 1080 cm^{-1} identifying an asymmetrical stretch (Si-O-CH_3). Transmittance at 810 cm^{-1} was detected confirming the presence of a symmetrical stretch and finally transmittance at 1260 cm^{-1} which represented a deformation (Si-CH_3). When compared with the spectra of control silicone rubber a high level of homology was evident. Light microscopy revealed the spin-coated rubber to be smooth and free of defects.

5.4.2 Parallel-plate flow chamber adherence assay with *Proteus mirabilis*

All surface treatments significantly reduced the adherence of *P. mirabilis* after 5 h (Figures 5.7 a-g and 5.8; Table 5.1; $P<0.05$). *Proteus mirabilis* adhered to control materials at an average of $42 \times 10^3 \pm 20 \times 10^3$ bacteria /mm^2 (n=21), compared with $20 \times 10^3 \pm 4 \times 10^3$ bacteria /mm^2 (n=21) for T4 material, which exhibited the greatest reduction in adherence. However, variations in adherence between the surface treatments were not significant indicating no relationship between surface hydrophobicity and adherence.

Figure 5.5: FT-IR spectrum of control silicone rubber

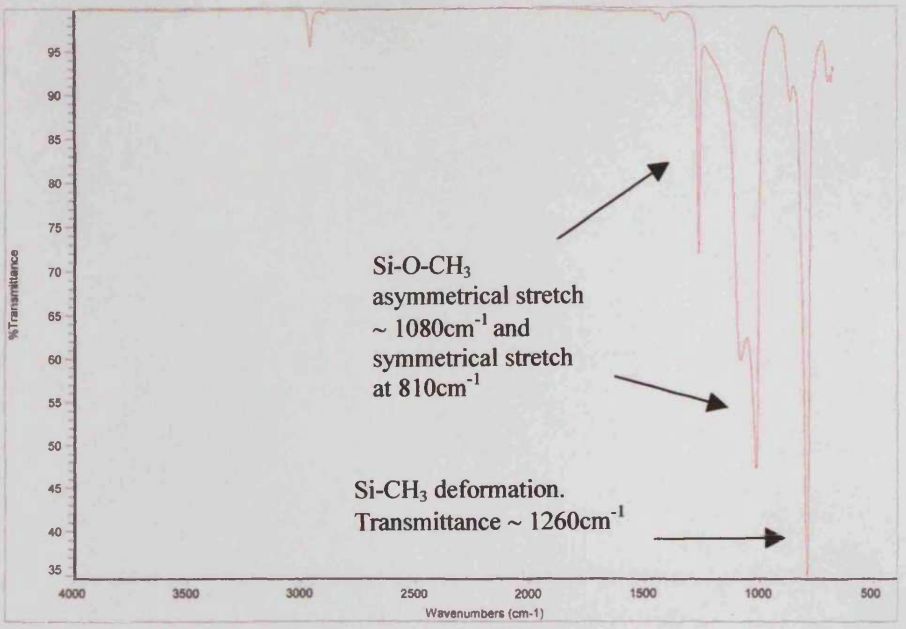


Figure 5.6: FT-IR spectrum of silicone rubber spin-coated onto glass. The spectra displayed the same transmittance peaks as that of silicone rubber (Figure 5.5 above)

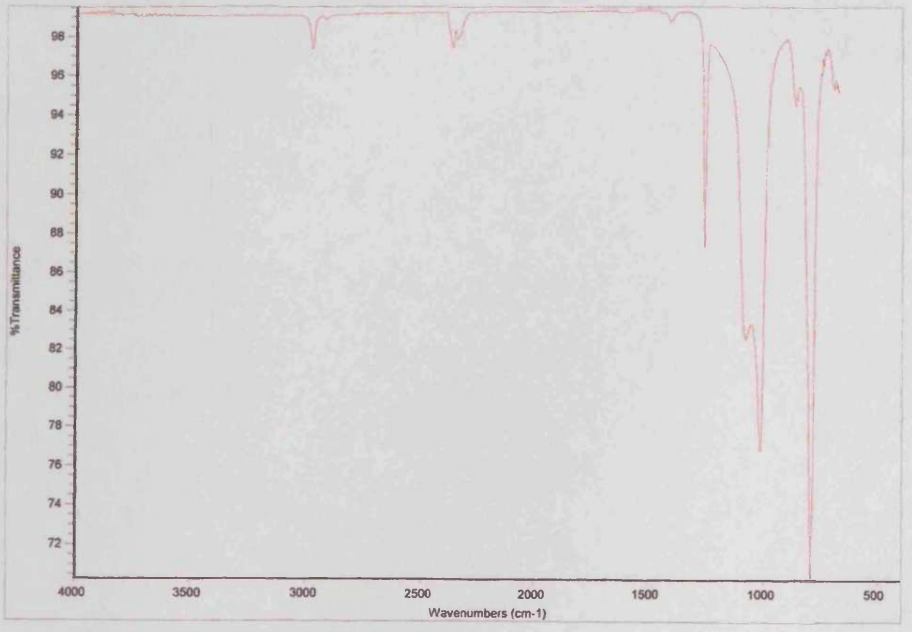


Figure 5.7 a-g: Adherence of *P. mirabilis* suspended in buffer to control silicone rubber and surface-treated materials after 300 min in a parallel-plate flow chamber.

Figure 5.7 a: Control material

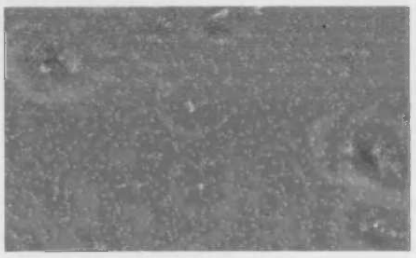


Figure 5.7 b: T1 material

Figure 5.7 c: T2 material

Figure 5.7 d: T3 material

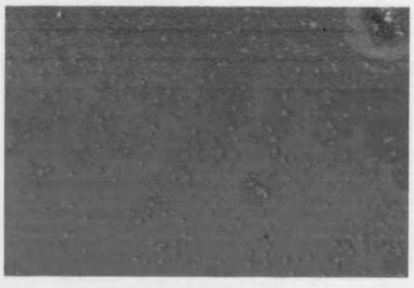


Figure 5.7 e: T4 material

Figure 5.7 f: T5 material

Figure 5.7 g: T6 material

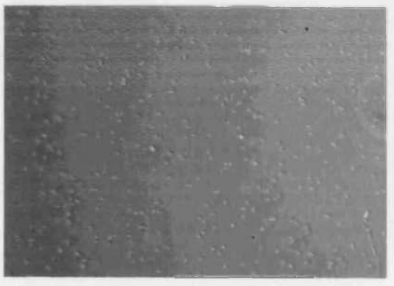


Table 5.1: *Proteus mirabilis* adherence to surface-treated silicone rubber after 300 min in the parallel plate flow chamber.

Surface treatment	Bacteria × 10^3 adhering/mm^2	Bacteria × 10^3 adhering/mm^2 after a passage of air through chamber
Control	42 ± 20	8 ± 3
T1	21 ± 5	5 ± 2
T2	25 ± 6	21 ± 3
T3	27 ± 8	6 ± 4
T4	20 ± 4	10 ± 2
T5	28 ± 8	19 ± 5
T6	29 ± 8	9 ± 9

Time-lapse images demonstrated that at each time point, the adherence of bacteria to control materials was higher than for treated surfaces (Figures 5.9, 5.10 a-g and 5.11 a-g). After the injection of air, significant numbers of the bacteria detached from the materials. In the case of the control, 3.2×10^3 /mm^2 bacteria were removed (8.0×10^3 retained, equivalent to 80.3% detachment). Similar levels of detachment occurred with all surface treatments apart from T1 and T3 where detachment was significantly greater ($P < 0.001$) (Table 5.1).

5.4.3 Parallel-plate flow chamber assay incorporating urine

Proteus mirabilis growth flourished in the presence of urine and the adherence of *Proteus* to the silicone rubber was much higher in urine than in its absence. After approximately 2 h, crystals began to develop in the flow chamber which caused more bacteria to become trapped within the chamber (Figure 5.12a). After 3 h, there was a large build up of crystals and *Proteus* within the chamber, making *Proteus* adherence difficult to determine (Figure 5.12b) and after 5 h, the crystals were visible macroscopically (Figure 5.12c). After 5 h, there was no significant difference between the percentage coverage of *Proteus*/crystals on the control silicone rubber and the surface-treated silicone rubber (Table 5.2; Control – 87 ± 18 %; T3 material – 99 ± 4 %; T4 material – 93 ± 14 %; T6 material – 100 ± 2 %). A passage of air through the flow chamber at the end of the experiment reduced bacterial coverage on control silicone rubber to 48 ± 14 % of the original amount. This amount was significantly higher than with treated material (T3 material – 12 ± 4 %; T4 material 12 ± 7 %; T6 – 1 ± 2 %).

Figure 5.8: Adherence of *P. mirabilis* to surface-treated silicone rubber in a parallel-plate flow chamber. Error bars represent standard deviations.

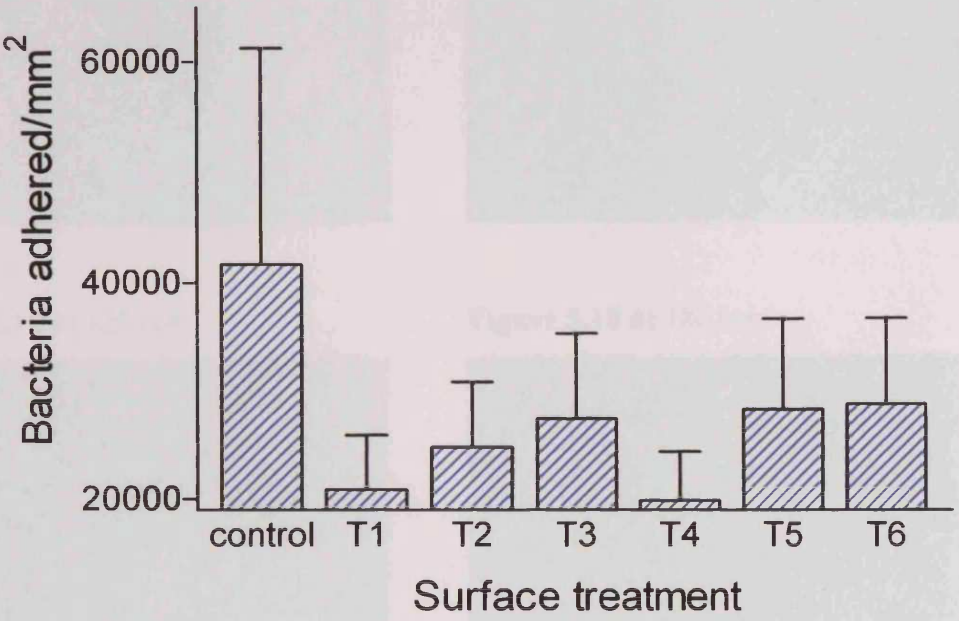


Figure 5.9: Bacterial adherence data derived from time-lapse images taken of spin-coated glass plates within a parallel-plate flow chamber over a period of 5 h. The decrease in adherence after 300 min is due to a passage of air through the flow chamber. Error bars represent standard deviations.

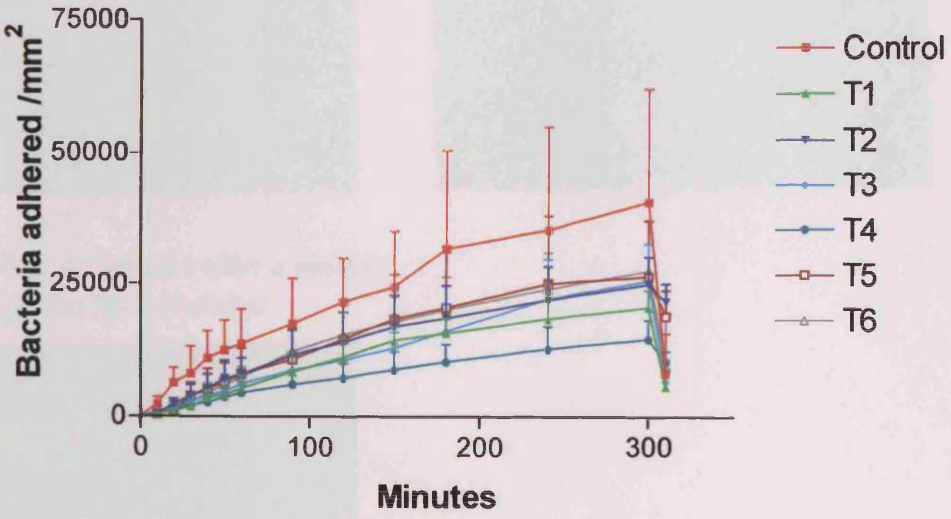


Figure 5.10 a-g: Adherence of *P. mirabilis* to control silicone rubber in the parallel-plate flow chamber over time

Figure 5.10 a: 10 min

Figure 5.10 b: 60 min

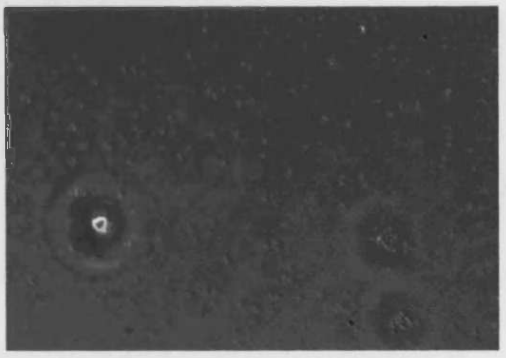


Figure 5.10 c: 120 min

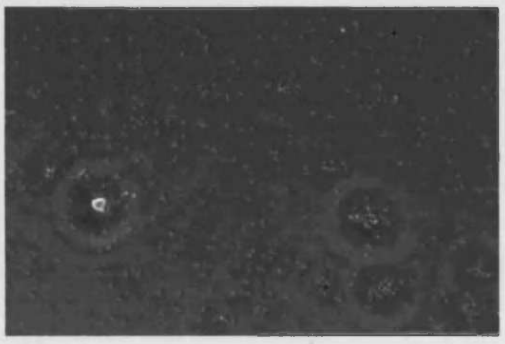


Figure 5.10 d: 180 min

Figure 5.10 e: 240 min

Figure 5.10 f: 300 min

Figure 5.10 g: Adherence after a passage of air through the flow chamber.

Figure 5.11 a-g: Adherence of *P. mirabilis* to T2 material in the parallel-plate flow chamber over time. (original mag. × 400).

Figure 5.11 a: 10 min

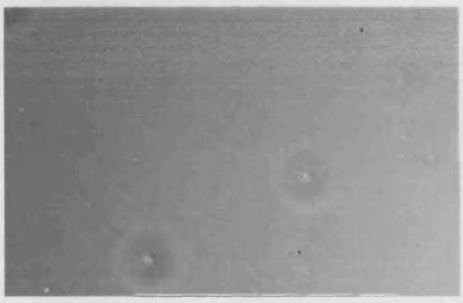


Figure 5.11 b: 60 min

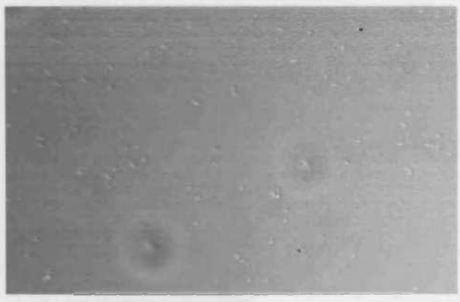


Figure 5.11 c: 120 min

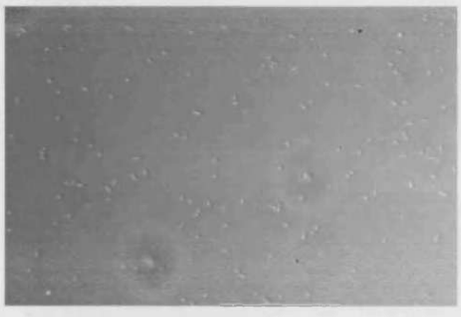


Figure 5.11 d: 180 min

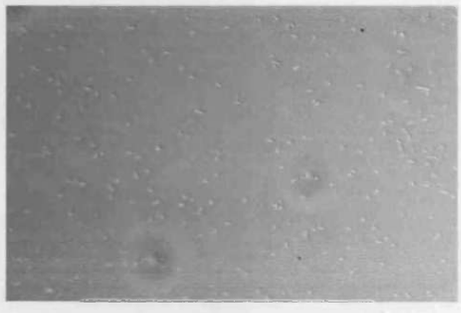


Figure 5.11 e: 240 min

Figure 5.11 f: 300 min

Figure 5.11 g: Adherence after a passage of air through the flow chamber.

Figures 5.12 a-c: *P. mirabilis* B2 suspended in human urine (× 400 original magnification). The dark patches are an accumulation of *P. mirabilis* and crystals formed due to an increase in the urine pH.

Figure 5.12a: 120 min

Figure 5.12 b: 180min

Figure 5.12 c: 300 min

5.4.4 Migration of *Proteus mirabilis* over surface-treated silicone rubber catheters sections

The motility assays demonstrated that all surface-treated silicone urinary catheters reduced the motility of *P. mirabilis* NSM42. The bacteria migrated across 100% of the untreated catheter sections but only 20% of the catheter sections treated with T1 which was the most successful at inhibiting *Proteus* motility. T3 treated material had the least inhibitory effect where *Proteus* motility was reduced by 23% (Figure 5.13).

5.4.5 Migration of *Proteus mirabilis* over urinary catheter sections

The motility of *P. mirabilis* varied between the different catheter types ($P<0.0001$; Figures 5.14 and Table 5.2). The most rapid migration occurred on the hydrogel-coated catheter sections on which the colonies travelled 27 ± 5 mm across the uninoculated section of the agar plate after 12 h. Slowest migration occurred on the all-silicone catheters where cells only managed to travel 12 ± 3 mm after migrating across the catheter bridge. The fastest migration occurred on the catheter with the roughest surface (hydrogel-coated).

5.4.6 Analysis of catheter surface roughness

The surface roughness of catheter sections is demonstrated by AFM and shown in Table 5.3 and Figures 5.15-5.22. AFM surface roughness analysis on the catheter sections revealed significant differences in roughness between the different catheter

Figure 5.13: Percentage of catheter sections that *P. mirabilis* migrated across to the uninoculated section of the agar plate within 18 h.

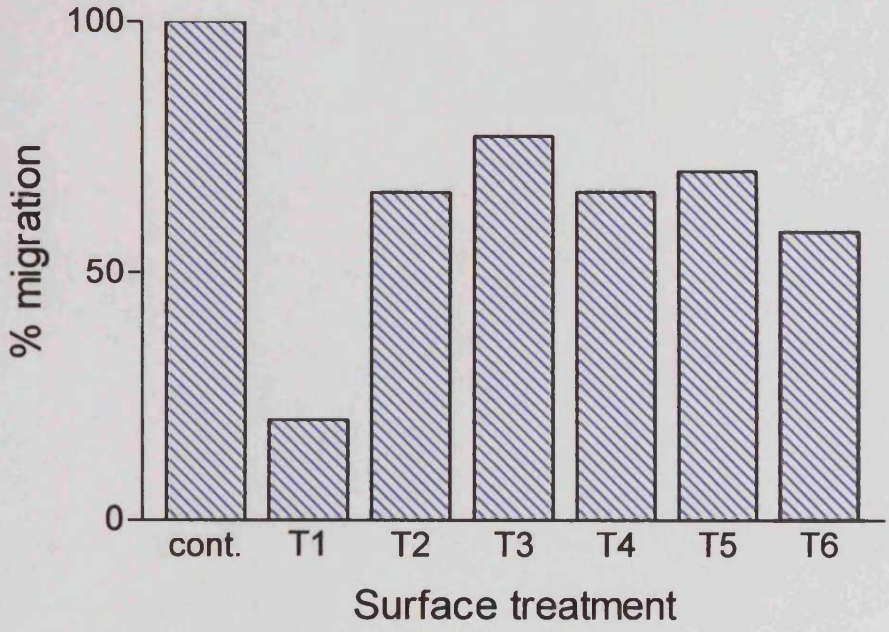


Figure 5.13: Motility assay demonstrating the migration of *P. mirabilis* across silicone elastomer-coated latex catheters to the other side of the agar plate. The control section of agar remains uninoculated demonstrating the migration of *P. mirabilis* across the catheters and not the exposed plastic.

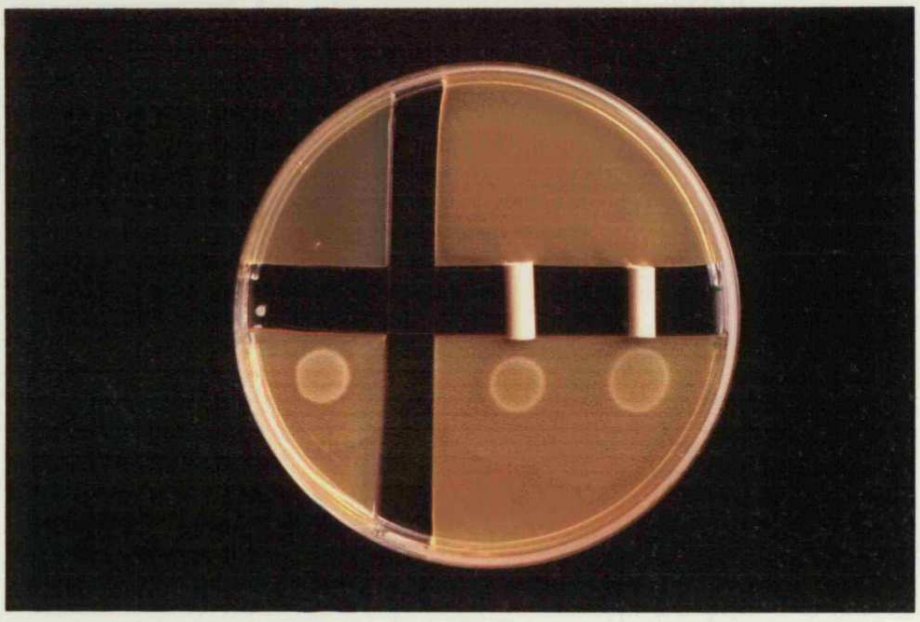


Figure 5.14: *P.mirabilis* migration across hydrogel/silver-coated latex catheters.

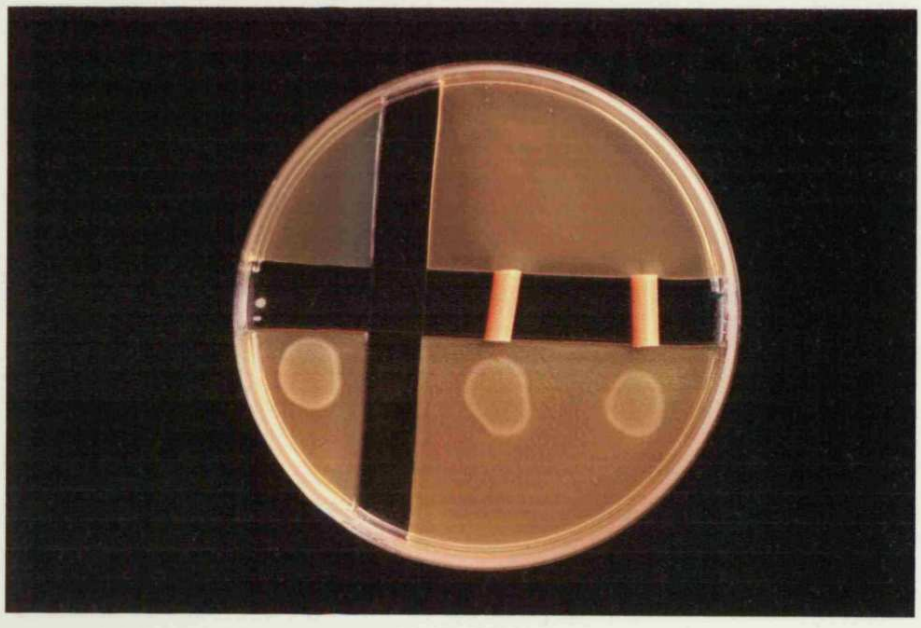


Table 5.2: Distance travelled by *P. mirabilis* NSM 42 in mm across uninoculated agar section after migrating across catheter bridge.

Catheter type used as a bridge	Distance travelled by bacteria after 12 hours (mm) ± SD
All-silicone	12 ± 3
Silicone-coated latex	18 ± 6
Hydrogel-coated latex	27 ± 5
Silver/hydrogel-coated latex	20 ± 9

Table 5.3: Catheter surface roughness derived from 1μm^2 AFM scans

Catheter type	Lumen surface roughness		Eyehole surface roughness	
	Ra ± SD (nm)	Rz ± SD (nm)	Ra ± SD (nm)	Rz ± SD (nm)
All-silicone	6 ± 2	99 ± 36	23 ± 7	189 ± 59
Silicone-coated latex	10 ± 5	154 ± 70	28 ± 27	281 ± 143
Hydrogel-coated latex	82 ± 48	763 ± 436	19 ± 10	206 ± 109
Silver/hydrogel-coated latex	32 ± 29	444 ± 244	47 ± 30	620 ± 246

Figure 5.15: AFM image of an all-silicone catheter eye hole surface using tapping mode (50 μm^2 scan size).

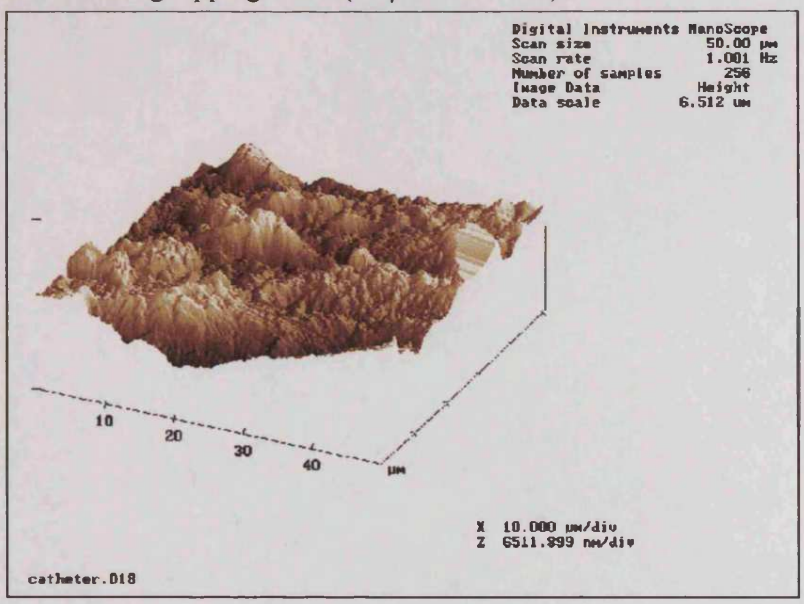


Figure 5.16: AFM image of all-silicone catheter lumen using tapping mode (50 μm^2 scan size).

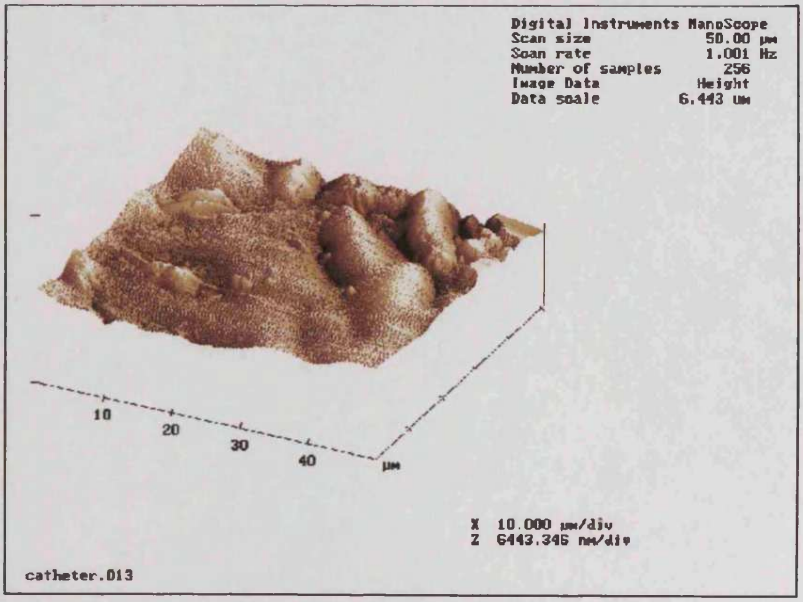


Figure 5.17: AFM image of a hydrogel catheter eyehole surface using tapping mode (50 μm^2 scan size).

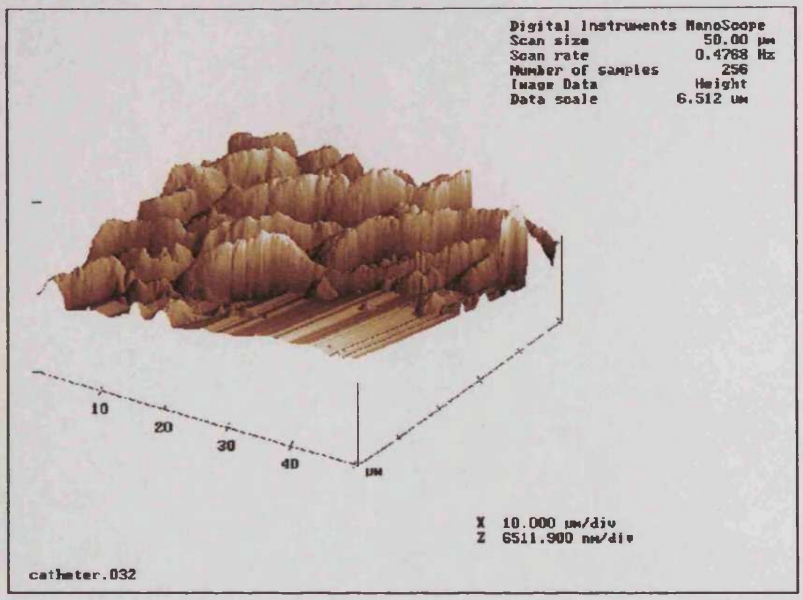


Figure 5.18: AFM images of a hydrogel catheter lumen using tapping mode (50 μm^2 scan size).

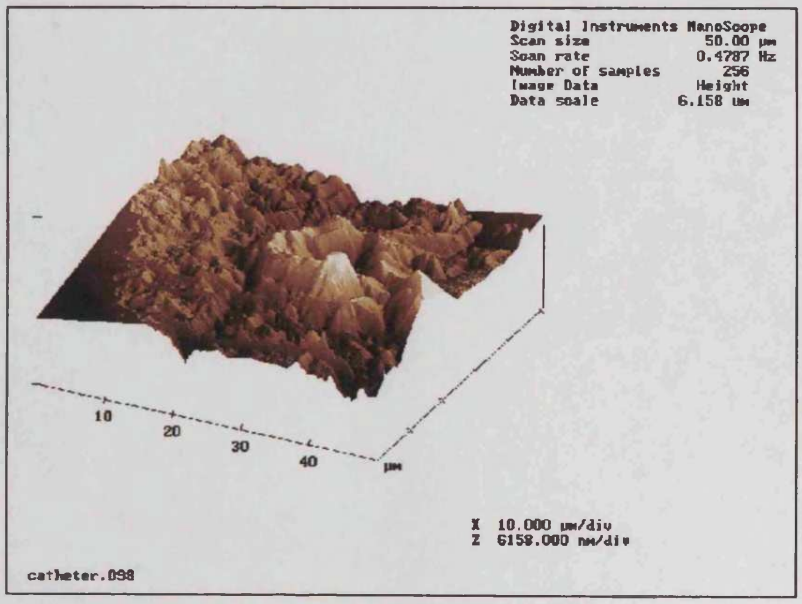


Figure 5.19: AFM image of a silicone elastomer-coated latex catheter eyehole surface using tapping mode (50 μm^2 scan size).

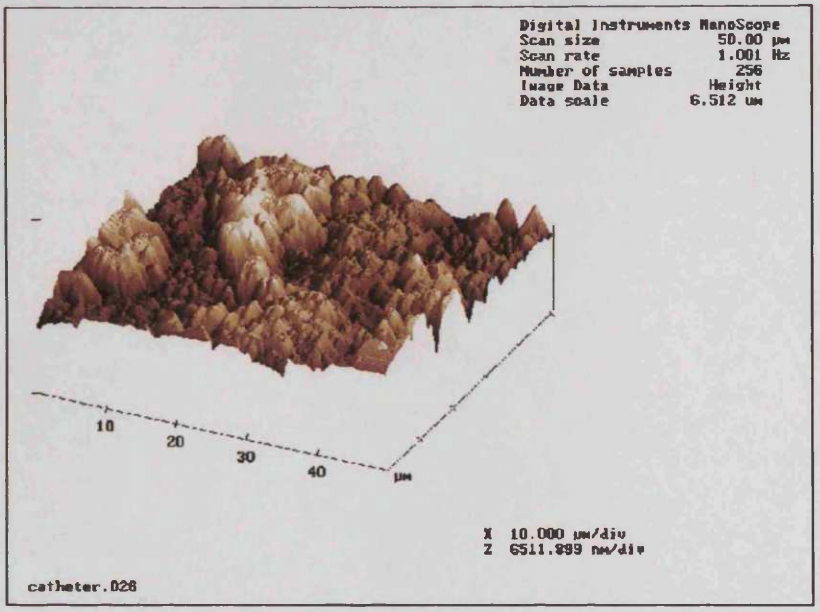


Figure 5.20: AFM image of a silicone elastomer-coated catheter lumen using tapping mode (50 μm^2 scan size).

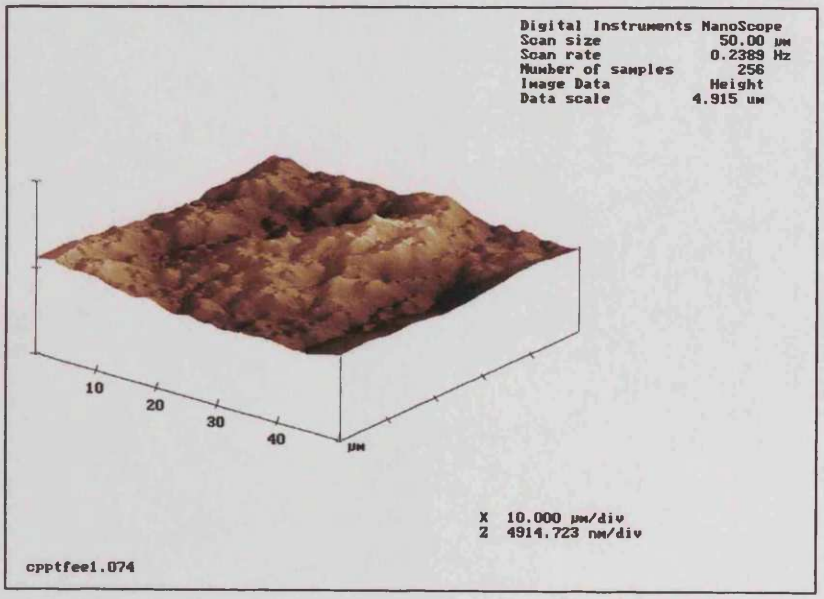


Figure 5.21: AFM image of a silver/hydrogel-coated catheter eyehole surface using tapping mode (50 μm^2 scan size).

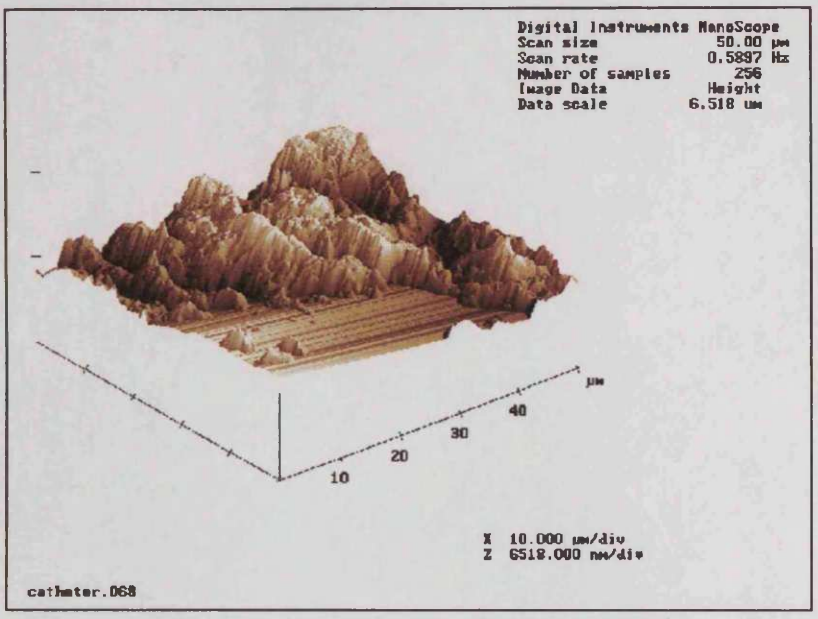
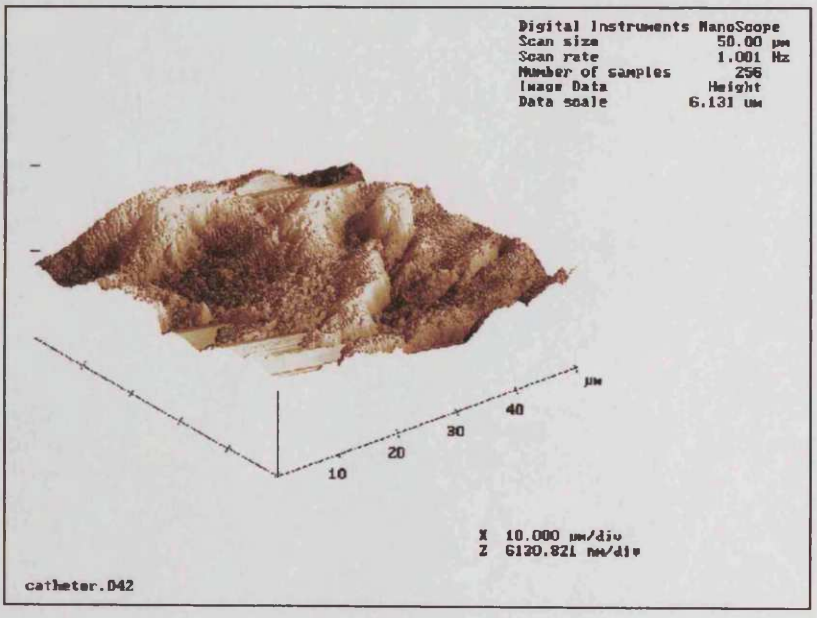


Figure 5.22: AFM image of a silver/hydrogel-coated catheter lumen using tapping mode (50 μm^2 scan size).



coatings (Figure 5.23; P< 0.001). The hydrogel-coated catheter eye-hole (hole at tip of catheter, which drains urine from bladder) and lumen were substantially rougher than that of the all-silicone catheter; on 1 μm^2 scans, the lumen and eyehole surface Ra values of the hydrogel-coated catheter were 82 ± 48 nm and 19 ± 10 nm respectively (n = 50) compared to Ra values of 6.3 ± 2.6 nm (lumen) and 23 ± 6.6 nm (eye-hole) (n = 50) for all-silicone catheters. The all-silicone and silicone-coated latex catheters proved to have the smoothest surfaces. The Ra value of the silicone-coated latex catheter lumen was 10.33 ± 5.43 nm (n = 50), and the Rz (Z-range) was 154 ± 70 nm (n = 50; scan size – 1 μm^2), although this roughness was not significantly different from that of the all-silicone catheter. The hydrogel and silver/hydrogel-coated catheters were both significantly rougher than the all-silicone and silver/hydrogel-coated latex catheters but were not significantly different from each another. The hydrogel and silver/hydrogel-coated lumens both had particularly high Rz values; 762 ± 435 nm (n = 50) and 443 ± 243 nm (n = 50) respectively compared to an Rz value of 188 ± 59 nm (n = 50) for all-silicone lumens (1 μm^2 scans). In addition there was a significant difference between the surface roughness of the lumens and eye-holes on the all-silicone and silicone-coated latex catheters where it was demonstrated that the eye-holes were rougher than the lumens (Figure 5.24).

Figure 5.23: Ra values of catheter lumens as derived from 1 μm^2 AFM scans. Error bars represent standard deviations. (Si-h = silver/hydrogel-coated, s-c = silicone-coated latex)

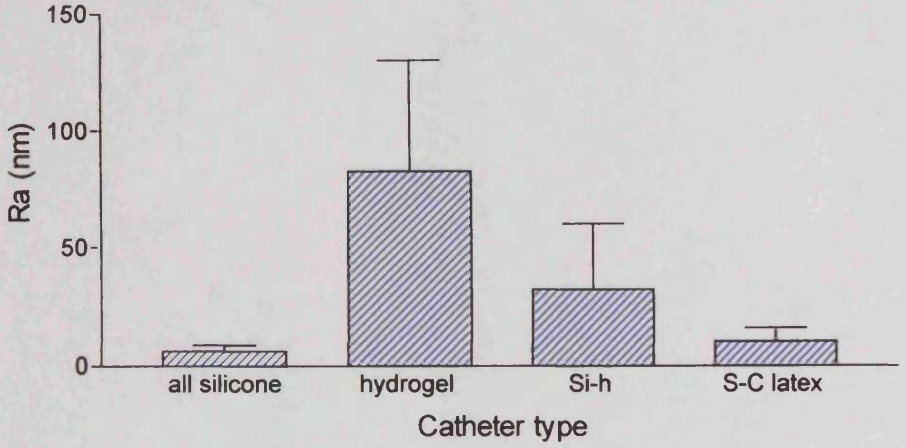
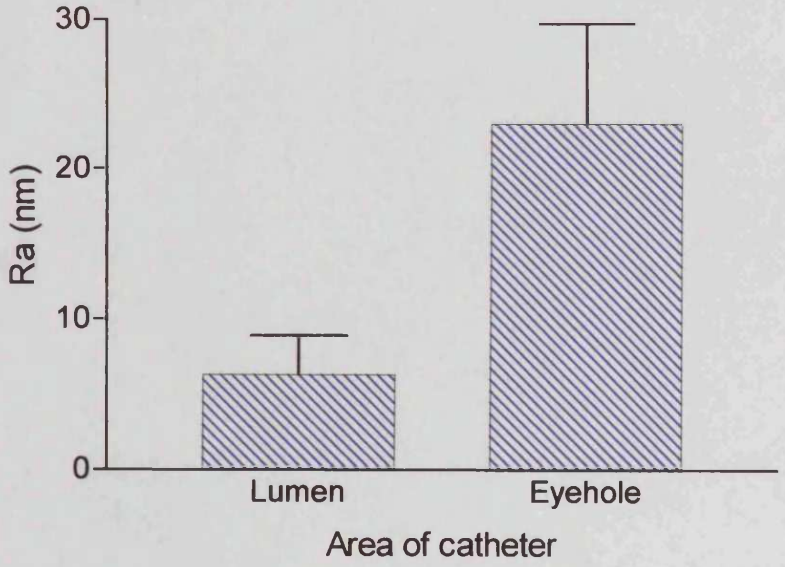


Figure 5.24 Differences in roughness between the all-silicone catheter lumen and eyehole as derived from 1 μm^2 AFM scans. Error bars represent standard deviations.



5.5 Discussion

CAUTIs and catheter blockage are common problems in long-term catheterised patients with limited access to healthcare providers. *Proteus mirabilis* is a significant cause of these infections and as a urease producing species, plays a major role in catheter blockage. Various measures have been implemented to help prevent such infections including the prophylactic administration of antibiotics. Attempts have also been made to produce catheters that prevent the colonisation of bacteria. However, these measures have largely proved unsuccessful in preventing infection.

The work described in this chapter aimed to assess the adherence and motility of *P. mirabilis* to the silicone rubber surface treatments, which have previously been found to inhibit the adherence of *C. albicans* (Chapter 3). Further work investigated the association between the surface topography of four commercially available catheters and *P. mirabilis* motility.

5.5.1 Silicone rubber spin-coating.

Silicone rubber was spin-coated on to the glass plates to be used in the parallel-plate flow chamber. The spin coating technique had proven more successful than initial methods involving the placement of silicone rubber films (0.5 mm thick) on to the glass slides. This earlier method proved unsuccessful as trapped air present in the films resembled *P. mirabilis,* making later enumeration of bacteria difficult. The spin coating technique allowed centrifugal forces to spread silicone rubber dissolved

in chloroform across the glass plates. The presence of silicone rubber was confirmed by FTIR (Fourier transform infra-red spectroscopy).

5.5.2 *Proteus mirabilis* adherence to surface-treated silicone rubber

The parallel-plate flow chamber was selected for assessing bacterial adherence to the silicone rubber as it had been successfully used by a number of researchers (Busscher *et al.*, 1997; Everaert *et al.*, 1998; Gottenbos *et al.*, 2002; Johnston *et al.*, 1997; Jones, 2001; Milsap *et al.*, 2001). As an *in vitro* adherence assay, it provides an accurate model of flow through an infected urinary catheter. The number of bacteria detected on the glass plate relates to the duration of a flow chamber experiment. Previous researchers have used experiments lasting several days in order to examine biofilm formation (Jones 2001) but in this study assays were only performed for 5 h to detect initial adherence to the plate surface. When using flow cells, some researchers have monitored the top plate of the flow cell for microbial adherence in the belief that this will rule out cell sedimentation (Cairns *et al.*, 2003). However, in this study, the flow of suspension through the chamber appeared to be sufficiently high to prevent sedimentation. The re-circulation of bacterial suspension through the flow cell during an experiment is a limitation of the technique as this would not occur *in vivo*. Despite this limitation, the flow cell technique used in this work, allowed real-time *in situ* observation of bacterial adherence and supplied a means of assessing the adherence to surface-treated silicone rubber without the occurrence of unwanted variables such as an air-surface interface.

The adherence of *P. mirabilis* B2 was reduced significantly on the surface-treated silicone rubber compared to the control. T4 material showed the greatest reduction in adherence although there was no significant difference between this and the other treatments. Although still statistically significant, the observed reductions did not appear to be as marked as those evident with *C. albicans* (Chapter 3, Table 3.2). There appeared to be no correlation between material surface hydrophobicity and bacterial adherence so similar mechanisms to those inhibiting *Candida* (Chapter 3, Section 3.5) may be involved *i.e.* physical mechanisms including the increased mobility of the surface chemical groups. Furthermore, since the inhibition occurred with both *Candida* and *Proteus* the effects appear non-specific and may add credence to the hypothesis of a physical inhibition mechanism. The reduced inhibitory effect of the surface treatments on the bacteria compared to the yeast may be due to relative physical sizes of the different microorganisms.

The passage of air through the flow cell at the end of each experiment demonstrated that the relative strength of the adhesive forces of surface-treated silicone rubber was similar to the control material, indicating that the surface treatments did not affect the binding mechanism once the bacteria had adhered.

5.5.2.1 Parallel-plate flow chamber assay incorporating urine

On suspension in human urine, the morphology of *P. mirabilis* changed. The bacteria became larger and elongated, possibly developing into swarmer cells. The bacteria also appeared to reproduce at a higher rate than cells within PBS. This was a limiting factor, as after 5 h, the population size of the bacteria was unknown.

Compared to *Proteus* suspended in PBS, the bacteria rapidly adhered to the silicone rubber and induced crystal formation. The surface-treated silicone rubber did not appear to have any influence over the level of *Proteus* adherence, which suggests that the presence of urine reduced the inhibitory effects of the treated-materials or changed the morphology of the *Proteus* in a manner that made them more adherent to the material surfaces. It must be taken into account however, that the population of bacteria after 5 h in urine was higher than in PBS so more cells would have adhered to the control.

5.5.3 Migration of *P. mirabilis* over surface-treated silicone rubber catheter sections

The strain *P. mirabilis* NSM42 (isolated from an infected urinary catheter) was selected for the motility assay due to its high propensity for forming swarmer cells. The modified surfaces reduced the motility of this strain compared with the control. The ability to form the swarmer cell phenotype remained unaffected as evident by the occurrence of these cell types on the agar adjacent to the catheter section. This indicates that surface treatment may have impeded the movement of the cells across the catheter in the same way that adhesion was inhibited. A second possibility is that on reaching the catheter, the cells inhibited from swarming onto the surface due to the presence of altered functional groups. Further work could investigate these hypotheses by performing time-lapse imaging and incorporation of urine (which hindered microbial adherence inhibition in the flow cell experiments). It is important to note, that during the growth and cell differentiation of a *Proteus* colony (lag phase) an extracellular polysaccharide slime is generated (Allison and Hughes, 1991)

which surrounds the colony and precedes it during migration. It is postulated that the slime is produced to aid surface migration (Stahl *et al.*, 1983) by enhancing surface fluidity and possibly influencing cell-cell interactions (Gygi *et al.*, 1995). This polysaccharide layer may be compromised by the presence of the altered functional groups on the treated surfaces.

5.5.4 Analysis of catheter surface roughness

AFM scans of catheter eyehole surfaces and lumens revealed significant differences in the roughness of the lumen and the eye-holes, with the eye-holes generally having higher Ra and Rz values. The eye-hole surface is a problem area in terms of microbial colonisation as demonstrated by Stickler *et al.*, (2002) who monitored artificial bladder models infected with *P. mirabilis* for areas of biofilm formation. In all cases, the catheter eye-hole was the first region to become encrusted and most blockages occurred within this area. Stickler *et al.*, (2003) demonstrated using SEM that the catheter eye-holes appeared to be rougher than the rest of the catheter and suggested this was the reason for increased colonisation. The results of the AFM roughness measurements in this thesis confirm these results. The reason for the irregular eye hole surfaces is due to the manufacturing methods which according to Stickler *et al.*, (2003), are out-of-date and only employed because of their low cost and convenience.

AFM measurements demonstrated that catheter surfaces were rougher than the surfaces of the silicone rubber produced in this thesis. The Ra of catheter lumen

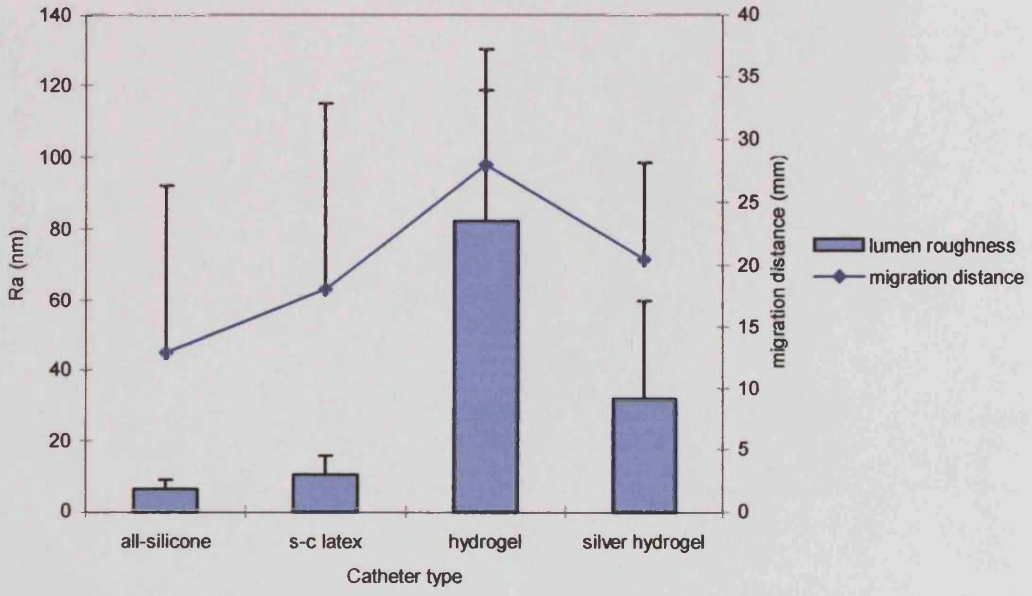
surfaces on 1 μm^2 scans ranged from between 10 and 75 nm whereas for silicone rubber, the values were between 4 and 6 nm. The increased roughness of the catheter surfaces may be aiding the colonisation of microorganisms.

Differences in roughness were also observed between the catheter types, with the hydrogel-coated and silver/hydrogel-coated catheters having significantly higher Ra and Rz values than the all-silicone and silicone-coated catheters. On exposure to water, the hydrogel layer of a catheter coated with hydrogel expands forming a gel–like layer which is designed for easy insertion (Cox *et al.,* 1989). It is therefore important to note that the topography of a hydrogel surface may vary on the addition of water.

5.5.5 Motility assay on urinary catheters

Motility assays revealed that *P. mirabilis* migrated faster across the hydrogel and silver/hydrogel-coated catheters. The ease of migration across the silver/hydrogel-coated catheter despite the antimicrobial properties of silver was not surprising as past research has demonstrated the inability of silver at preventing bacterial colonisation on urinary catheters (Chapter 1, Section 1.8.1.1). There appears to be a correlation between surface roughness and speed of migration across the catheter sections suggesting that the bacteria migrate across rougher surfaces more easily (Figure 5.25). However, the chemistry of the different surface coatings must also be taken into account. For example, hydrogel has a different chemistry to silicone rubber and it is more hydrophilic and the presence of silver on a catheter will also alter the surface chemistry.

Figure 5.25: The relationship between catheter surface roughness and rate of *P. mirabilis* migration across the catheter section. This was determined by the distance the bacterial colonies travelled in 12 h once arriving onto the un-inoculated side of the agar plate. Error bars represent standard deviations.



Conclusions

- Silicone rubber was successfully coated on to glass plates using a spin coating technique
- All surface treatments tested (T1-T6) showed a significant reduction in adherence of *P. mirabilis* B2 suspended in PBS compared to the control. In the presence of urine however, no difference was apparent.
- The surface-treated materials had no effect on the strength of the *P. mirabilis* adherence to the silicone rubber in the presence of PBS. In the presence of urine, the surface treatments reduced the bacterial strength of attachment.
- The presence of urine produced more extensive bacterial adhesion and also induced crystal formation which could be observed macroscopically.
- The surface-treated silicone rubber reduced the motility of *P. mirabilis* NSM42 across catheter sections by up to 80 %.
- All catheters tested had a higher roughness than the new silicone rubbers, with hydrogel-coated having the highest average roughness. Catheter eye-holes had rougher surfaces than catheter lumens.
- *Proteus mirabilis* migrated more quickly over the rougher catheter surfaces (hydrogel and silver/hydrogel-coated) indicating that an increased surface roughness may be favourable to *P. mirabilis* swarmer cell migration.
- The mechanism for the inhibition of bacterial adherence and motility appears to be physical rather than chemical, which confirms the hypothesis suggested in Chapter 3 that the increased motility of the surface functional groups on the treated silicone rubber are inhibiting adherence.

Chapter 6

General discussion

6.1 Introduction

Biomaterials are used extensively in healthcare and are a fundamental part of modern medicine. Silicone rubber is a commonly used biomaterial due to its biological inertia and ideal mechanical properties (Chapter 1). Silicone rubber is often used to fabricate medical devices including urinary catheters, denture-soft liners and voice box prostheses. However, mainly due to the non-ablative nature of biomaterials, they often become colonised by a microbial biofilm and subsequently provide a site for infection and/or medical device impairment. To reduce the problem of biofilm development on medical devices, the construction of a material that inhibits microbial adherence would be desirable. With this in mind, the aim of this study was to chemically modify the surface of silicone rubber to reduce initial microbial adherence.

Chemical modification of the silicone rubber surface was the chosen approach to generate the novel materials as it was envisaged that this would not affect the bulk properties of the silicone rubber. This approach taken was not reliant on antimicrobial incorporation, which can influence the bulk properties of the rubber and induce microbial resistance.

In the design of a surface inhibitory to microbial adherence, problems immediately arise when consideration is given to the diverse spectrum of microorganisms that exist. Accompanying this diversity is the variation in cell wall structures, compositions and receptor types. The plethora of microbial cell surface characteristics result in contrasting cell behaviour when in contact with biomaterial

surfaces. Accounting for the variation in cell response is obviously difficult within a single material. Therefore, this project focused on two distinct microorganisms that are prevalent in causing medical devices related infections. The microorganisms were *Candida*, which causes medical device dysfunction and infection within the oral and oropharyngeal cavities and *Proteus mirabilis*, which is a coloniser of urinary catheters. *Candida albicans* is a well documented coloniser of oral and oropharyngeal medical devices such as denture-soft liners (Waters *et al.*, 1997) and voice box prostheses (Elving *et al.*, 2003). *Proteus mirabilis* is a frequent coloniser of urinary catheters and due to its urease producing capability, leads to urine crystallisation. A potential consequence of urine crystallisation is blockage of the catheter (Chapter 5).

6.2 Surface treatment process

Biomaterial surface hydrophobicity has an influence on initial microbial adherence as does cell surface hydrophobicity (Chapter 1). The aim of this study was to investigate the effect of silicone rubber surface hydrophobicity on microbial adherence and this was achieved by altering the surface of silicone rubber to produce a range of materials with varying hydrophobicities. Other surface features thought relevant to adherence were functional group chain length and mobility. Consequently, these were also altered. The approach selected to generate the new silicone rubbers was based on argon plasma discharge treatment of the silicone rubber to produce a chemically reactive surface. The advantage of this approach was that the bulk properties of the 'new' materials would remain unaltered. To introduce new surface chemistries, the reactive silicone samples were immersed in eight

different silane solutions. Silanes are difunctional molecules possessing reactive groups at one end, which couple to the activated silicone surface and specific functionalities at the other end, which alter the surface chemistry. Selection of these silanes was based on their functional group hydrophobicity and chain length and also their previously reported abilities to inhibit protein adsorption (Chapter 2).

This method successfully provided a range of chemically and physically altered surfaces that XPS revealed to be relatively stable over a period of six months. The surfaces varied in their functional group chemistry and chain length and consequently provided altered surface hydrophobicity (although not to the expected extent), surface chemistry and surface mobility. Surface topography was not notably altered by chemical modification as determined by AFM. Alterations in surface mobility were not determined although others have suggested that such changes can arise using similar surface treatment techniques (Everaert *et al.*, 1998b) and the presence of a siloxane bond will increase mobility as this type of bond has more flexibility. The surface treatment process did not influence silicone rubber acrylic bonding or polymer water absorption.

6.3 Considerations when assessing microbial adherence to a surface

Following surface characterisation, the surface-treated silicone rubber was subjected to four different microbial adherence assays, one of which was performed at Manchester Metropolitan University and one at Groningen University in Holland. By assessing the materials using a variety of assays, enhanced confidence in the performance of the materials was obtained. Inconsistencies can arise in individual

assays due to the different assaying techniques available (*e.g.* Parallel-plate flow chamber, cylindrical channel, rotating disk, stagnation point flow collector) and also due to human error. The assays performed in this study produced large standard deviations, which may be expected when performing adhesion assays due to the nature of microorganisms. This has been noted in other studies (Waters *et al.,* 1997; Nikawa *et al.,* 1989; Samaranayake *et al.,* 1980).

Another important variable when performing microbial adhesion assays is material surface roughness. The work in this thesis aimed to eliminate this variable by fabricating silicone rubber from a smooth aluminium mould rather than a commonly used plaster based mould. AFM assessment of the silicone rubber surface topography demonstrated that roughness levels were below those deemed to affect microbial adherence (Bollen *et al.,* 1997).

6.4 Adherence assays

The adherence assays were performed both in the presence and absence of conditioning films. Salivary conditioning films were incorporated into the *Candida* assays while urinary conditioning films were incorporated into the *P. mirabilis* assays. The adherence assays without the presence of a conditioning film revealed a drastically reduced level of candidal adherence on all the surface-treated materials. For example, T5 material showed 55 times less *C. albicans* adherence than the control material. The adherence of *P. mirabilis* was also significantly reduced on all surface-treated materials, with T1 displaying half the adherence of the control. This reduction in adherence was not as marked as that for *C. albicans*, which may be due

to the different surface properties of the two microbes and/or the smaller size of *P. mirabilis* cells. In addition to adherence assays, *P. mirabilis* motility assays were also performed on surface-treated silicone rubber urinary catheters. The motility of *P. mirabilis* is a virulence factor that aids its colonisation on catheters by allowing the bacterium to migrate along the external wall and lumen of the catheter. All surface-treated catheters displayed a reduction in *P. mirabilis* motility. The most successful material was T1, which reduced the migration of *P.mirabilis* by 80%.

In the presence of a salivary conditioning film on the material, the adherence of *C. albicans* was higher, although this increase remained lower than the control on several of the treated materials, (T2 and T5 materials). The adherence of *P. mirabilis* in the presence of urine, was also higher but there was no difference between the treated materials and the control. Overall, the best performing surface treatments were T5 material, which displayed the highest degree of candidal inhibition and T1 material, which displayed the highest degree of *P. mirabilis* adherence inhibition (in the absence of urine) and motility inhibition. The same material did not inhibit both microorganisms to the same extent, which suggests a level of microbial specificity.

6.5 Adherence mechanisms

The assessment of microbial adherence to a range of surfaces varying in functional group chemistry, chain length and mobility, led to the hypothesis that increased surface mobility had an inhibitory effect on microbial adherence. This hypothesis has not generally been suggested by the academic community. It is generally accepted that changes in adherence are the result of varying surface

hydrophobicity.(refs) However, in this study, the hydrophobicities of the silicone rubber surfaces were not notably altered by surface treatment, which suggests other mechanisms played a role. Increased surface functional group length has been suggested as a mechanism of inhibition (Everaert *et al.*, 1998a), which was also discounted in this study.

Microbial adherence mechanisms become more complex in the presence of conditioning films due to the addition of proteins which can act as receptors for microbes and can mask the underlying surface characteristics. The presence of conditioning films (derived from saliva and urine) on the materials developed in this study generally caused an increase in microbial adherence although adherence to several of the treated materials (T4 and T5) remained significantly lower ($P<0006$ and $P<0.0001$ respectively) than the control. An explanation for this persistent inhibitory effect, even in the presence of saliva was sought. It was assumed that a protein from parotid saliva (proline-rich protein – PRP), which is known to bind to *C. albicans* (O'Sullivan *et al.*, 1997) was involved. This protein was isolated using gel filtration and an adherence assay was performed in the presence of PRPs. This demonstrated that inhibition or deactivation of this protein was not the mechanism of candidal inhibition.

6.6 Surface roughness as a contributing factor to urinary catheter infection

A second facet to this study was the investigation into the relationship between the topography of different catheter types and the subsequent motility of *P. mirabilis*. A number of catheters are commercially available with different catheter coatings,

aimed at providing easy insertion (*e.g.* hydrogel catheter) and antimicrobial properties (*e.g.* silver-coated). It is widely acknowledged that the currently available catheters containing antimicrobial coatings are unsuccessful at preventing infection in the clinical setting (Bayston, 2003). In addition, despite the potential benefits of 'easy insertion' catheter coatings, such as hydrogel, research has so far failed to assess the effects of the roughness of these coatings on microbial motility. The work undertaken in this study describes a positive correlation between catheter surface roughness and *P. mirabilis* motility rate. The highest rate of bacterial motility occurred on the hydrogel and silver/hydrogel-coated catheters, which had the highest Ra values. Consideration must however be given to the added variable of the surface chemistry of the coating. This finding is of clinical importance and suggests that catheter manufacturers should perhaps concentrate their efforts on the surface topography of catheters instead of various antimicrobial coatings that may create more bacterial colonisation through increased roughness.

As these findings are of clinical importance, particularly in the case of candidal adherence inhibition, it would be of benefit to continue this area of research in order to develop a final material that could be utilised in the clinical setting. To reach this point, further investigation may be needed to improve the stability of the surface treatment. It should be noted, however, that the treatment was still detected on the surface after six months. Possible measures to achieve a more stable surface could be to expose the silicone rubber to the plasma discharge treatment for a longer period in order to produce more activated groups or to use more concentrated solutions of the silane. Both of these measures could increase the functional group density on the surface. It would also be useful to further investigate the mechanism by which the

developed silicone rubber surfaces inhibit adherence, as this type of treatment could then be applied to the inhibition of other microorganisms. The final stage of this work, which would conclude the study would be to surface-treat voice box prostheses and denture soft liners and assess their performance *in situ.*

6.7 Concluding comments

This study has produced a number of novel silicone rubber surfaces that considerably reduce the adherence of *Candida* species and *P. mirabilis in vitro.* When applied to silicone rubber urinary catheters, the surface treatments also reduced the motility of *P. mirabilis* by up to 80%. Furthermore, several of these surface treatments (T4 and T5) remained successful at reducing candidal adherence even in the presence of a salivary conditioning film. These findings are of clinical importance and further work should assess the performance of these novel surfaces on medical devices *in situ.*

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