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Factors affecting in vitro adherence of ureteral stent biofilm isolates to polyurethane

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Abstract

Adherence of bacteria to biomaterials is the first stage in the development of a device-related infection. The adherence of bacterial cells to biomaterials may be influenced by surface characteristics of the cell, its growth conditions and the biomaterial surface chemistry. Following growth in human urine, the cell surface hydrophobicity and zeta potential of two ureteral stent biofilm isolates, *Enterococcus faecalis* and *Escherichia coli*, were significantly altered. In addition, the adherence of human urine-grown *Enterococcus faecalis* and *Escherichia coli* to polyurethane was significantly increased by up to 52.1% and 58.6%, respectively. Treatment of the polyurethane with human urine rendered the polymer surface more hydrophilic (mean advancing water contact angle reduced from 97.59° to 26.37°). However, organisms grown in human urine showed less adherence (up to 90.4%) to the treated polymer than those grown in Mueller-Hinton broth. The results presented in this study indicate that in vivo conditions should be simulated as far as possible when carrying out in vitro bacterial adherence assays, especially if assessing novel methods for reduction of adherence. © 1997 Elsevier Science B.V.

Keywords: Bacterial adherence; Hydrophobicity; Zeta potential; Ureteral stent; Human urine; Polyurethane

1. Introduction

The ureteral stent is a synthetic polymeric biomaterial, primarily fabricated from either polyurethane or silicone, which is designed to be retained within the urinary tract whilst conducting urine flow from the renal pelvis to the bladder. As with implanted biomaterials, the problem of bacterial attachment leading to device-related infection is associated with the use of ureteral stents. Infections associated with implanted biomaterials are typically caused by previously harmless skin commensals, or nosocomially acquired bacteria (Tunney et al., 1996). The infection is characterised by a low inoculum size and its persistence despite antimicrobial therapy. After initial attachment the infecting organisms typically form mi-

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crocolonies surrounded by an exopolysaccharide matrix which may provide resistance to antimicrobial agents. These microcolonies then establish a microbial biofilm on the biomaterial surface. In a recent clinical survey, the most common microbial colonisers of indwelling ureteral stents were *Enterococcus faecalis* and *Escherichia coli* (Keane et al., 1994).

The ability of bacterial cells to attach to biomaterial surfaces is dependent on the physicochemical characteristics of the bacterial cell, the bacterial growth conditions and the biomaterial surface. The relative hydrophobicity and surface charge of the organism play an important part in its attachment to the material surface, particularly as strong hydrophobic forces bring the bacterium to within 2–3 nm of the biomaterial. At these distances bacterial cell structures such as fimbriae can form strong links with the substratum. Altering growth conditions may affect bacterial adherence, for example, it was shown that the adherence of a ureteral stent biofilm isolate, *Enterococcus faecalis*, to polyurethane was increased by culturing the organism in an atmosphere enriched with 5% CO_2 (Bonner et al., 1995).

The nature of the biomaterial surface also influences bacterial adhesion. Reid et al. (1991) found that *Lactobacillus acidophilus* tended to preferentially adhere to hydrophilic materials than hydrophobic materials. After insertion of a stent in the urinary tract, host macromolecules are adsorbed onto the surface of the biomaterial in a process described as 'conditioning film formation' (Reid et al., 1992). The authors described the formation of a conditioning film onto a silicone latex catheter both in vivo and in vitro. These films were analysed by X-ray photoelectron spectroscopy and in comparison to the spectrum for the unused catheter, the spectra for retrieved catheters showed increased levels of carbon and nitrogen and the presence of small levels of calcium and phosphorous. The authors asserted that the formation of this film must impact on the adhesion process as the surface chemistry of the catheter material was altered. Carballo et al. (1991) showed that adsorption of albumin and fibrinogen onto polymeric surfaces affected subsequent staphylococcal adhesion.

This study aimed to investigate the effect of differing growth media on the surface properties of two organisms isolated from indwelling ureteral stents, and on their ability to adhere to polyurethane. The role of biomaterial conditioning film formation on the adherence of the isolates to polyurethane and on the polymer surface characteristics were also examined.

2. Materials and methods

2.1. Microorganisms

Enterococcus faecalis and *Escherichia coli* isolates recovered from microbial biofilm present on retrieved ureteral stents (Keane et al., 1994) and retained within the School of Pharmacy laboratories were used.

2.2. Cell surface hydrophobicity determination

The method used to determine cell surface hydrophobicity (CSH) is based on that detailed by Rosenberg et al. (1980), and is known as the bacterial adherence to hydrocarbons (BATH) technique. The organic phase used was *p*-xylene (Aldrich Chemical Co., Gillingham, Dorset, UK).

Bacterial isolates were cultured in Mueller-Hinton broth (MHB) or in human urine (HU) in an atmosphere containing 5% CO_2 to late logarithmic/early stationary phase and then harvested by centrifugation ($3000 \times g$, 10 min). On removal of the supernatant, pellets were washed with 10 ml of phosphate buffered saline (PBS) and centrifuged for a further 10 min at $3000 \times g$. The resulting pellet was resuspended at OD_{540} to 0.7 with PBS. Microbial suspension, 4.8 ml, was added to 1 ml volumes of xylene and vortexed for 30 s to ensure thorough mixing of the two phases. Phase separation was allowed to occur and the lower aqueous phase removed after 20 min. The absorbance of this phase was measured and the percentage absorbance of this phase relative to the suspension calculated. Five replicates were performed for each organism to enable statistical comparison of the results.

2.3. Determination of zeta potential

Early stationary phase bacterial cultures grown in MHB or human urine were centrifuged, washed three times with sodium phosphate buffer (pH 7.2) and resuspended in buffer to 0.3 at OD_{540}. This optical density was found to provide best reproducibility of zeta potential measurements. Zeta potentials were determined using a Malvern Zetasizer IV (ZET 5104, Malvern Instruments, Malvern, UK). A minimum of ten readings were recorded per sample with a field strength of 10–20 V cm^{-1}, electrode spacing 50 mm and dielectric constant 78.54. Zeta potentials were measured to a precision of ± 1 mV for all isolates.

2.4. Polyurethane contact angle measurement

Rectangular strips ($50 \times 10 \times 0.45$ mm) were cut from a polyurethane sheet (Vas-Cath Inc., Ontario, Canada) and immersed in either PBS or human urine for 24 h at 37°C before contact angle analysis. All the sections were stored in an incubator equilibrated to 5% CO_2.

Advancing and receding contact angles of the polyurethane were determined in triplicate by the Wilhelmy plate technique using a CAHN Dynamic Contact Angle Analyser, DCA 312 which was interfaced with a personal computer. The wetting medium used was high-performance liquid chromatography grade water. The surface tension of the water was obtained by measuring its contact angle onto a glass coverslip which had been cleaned by gentle flaming. The value obtained for the surface tension of the water was then used in determination of contact angles onto the biomaterials at zero depth of immersion.

2.5. Radiometric assay of bacterial adherence to polyurethane

The radiometric assay described by Gorman et al. (1993) was used to study the bacterial adherence to ureteral stent biomaterials.

Briefly, one colony of each isolate was inoculated into 100 ml MHB or human urine containing 50 μl [^{3}H]thymidine (1 mCi/ml, Sigma Chemicals, UK) and incubated for 18 h at 37°C in an atmosphere containing 5% CO_2. The suspension was centrifuged ($3000 \times g$, 10 min) washed twice in cold PBS and resuspended at OD_{540} to 0.7 with PBS. A 1 ml sample of the labelled suspension was serially diluted in PBS and the viable count after subsequent incubation on Mueller-Hinton agar at 37°C for 24–48 h.

Scintillation cocktail (10 ml, Optiphase 'Hi-Safe 2', LKB Scintillation Products, UK) was vortex-mixed with 1 ml radiolabelled bacterial suspension for 30 s and the radioactivity counted in a liquid scintillation system (1215 Rackbeta II liquid scintillation counter, LKB, UK). Five vials were counted and the labelling efficiency was calculated by dividing the viable count by the average disintegrations per minute. Five sections of PU (1 cm^2) were pre-soaked in PBS for 24 h, rinsed in cold PBS then incubated with gentle shaking in 2 ml radiolabelled bacterial suspension in McCartney bottles. At specified time intervals (2, 4, 6, 8 and 24 h) the sections were removed with sterile forceps and gently washed five times in cold PBS to remove any non-adherent bacteria. Prior experimentation confirmed no significant death or replication of the labelled organisms over a 24 h period in PBS. The final washing was counted to ensure that it was free from radioactivity. Each piece was then sonicated separately for 2 h and vortexed for 5 min in 5 ml PBS to ensure release of the radiolabel from the adhered bacteria. A 1 ml sample of each solution was then withdrawn and vortexed for 30 s in scintillation cocktail. The radioactivity of this mixture was then counted as before. The number of adhered bacteria on each section was obtained by dividing the DPM value for the section by the labelling efficiency. The percentage of the initial viable count of the labelled suspension adhering to the polyurethane sections was then calculated.

2.6. Effect of conditioning film formation on adherence to polyurethane

Isolates grown either in MHB or human urine in an atmosphere containing 5% CO_2 were labelled, harvested and resuspended as described above. Polyurethane were soaked in human urine for periods of 2, 4, 6, 8 and 24 h. The

Table 1
Effect of growth in Mueller-Hinton broth (MHB) and human urine (HU) condition on cell surface hydrophobicity and zeta potential of biofilm isolates (± S.D.)

	Cell surface hydrophobicity[a]		Zeta potential (mV)	
	MHB	HU	MHB	HU
Escherichia coli	71.58 ± 0.96	61.14 ± 0.59*	−35.16 ± 0.14	−28.5 ± 0.30*
Enterococcus faecalis	42.82 ± 1.75	70.96 ± 1.02*	−35.97 ± 0.10	−24.8 ± 0.50*

[a] Cell surface hydrophobicity values quoted as % bacterial cells retained within the aqueous layer in the BATH assay.
* Statistically significant result ($p < 0.05$).

sections were then incubated with the radiolabelled bacteria for 6 h, removed, washed and counted as before.

2.7. Statistical analysis

Statistical analysis was performed using one-way analysis of variance ($p < 0.05$ denoting significance).

3. Results

Table 1 shows the BATH values determined for the two isolates chosen. When grown in MHB, the *Escherichia coli* isolate remained within the aqueous layer, indicating that it was hydrophilic in character. In contrast, the *Enterococcus faecalis* isolate tended to move into the organic phase, indicating its hydrophobic character. Incubation in human urine rendered *Escherichia coli* more hydrophobic, whilst the *Enterococcus faecalis* isolate became more hydrophilic.

Both isolates had negative zeta potentials after incubation in MHB. Incubation in human urine significantly decreased the negativity of this value for both isolates (Table 1).

Table 2 shows the advancing and receding water contact angles for polyurethane treated with PBS and human urine. The polyurethane treated with human urine showed a significantly lower advancing and receding contact angle than that treated with PBS. This result indicates that exposure of polyurethane to human urine rendered the surface of the material more hydrophilic.

Fig. 1(a,b) show the adherence of the isolates grown in MHB and human urine to polyurethane. The *Enterococcus faecalis* isolate adhered to the polyurethane in substantially greater numbers than *Escherichia coli*. When the isolates were cultured in human urine, their adherence to polyurethane was significantly increased compared to their adherence when grown in MHB.

Fig. 2(a,b) show the adherence of the isolates grown in MHB and human urine, to polyurethane coated with a human urine conditioning film. Again, *Enterococcus faecalis* showed substantially greater adherence to the polyurethane than *Escherichia coli*. However, adherence of both isolates grown in human urine was significantly reduced by polyurethane pretreatment with human urine.

4. Discussion

The isolates used in this study were cultured in an atmosphere equilibrated with 5% CO_2 as this atmosphere is regarded as simulating physiologi-

Table 2
Advancing and receding contact angles of polyurethane treated with PBS and human urine (± S.D.)

Water contact angle (°)			
PBS		HU	
Advancing	Receding	Advancing	Receding
97.59 ± 0.85	45.14 ± 1.14*	26.37 ± 0.41	25.42 ± 0.37*

* Statistically significant result$p < 0.05$).

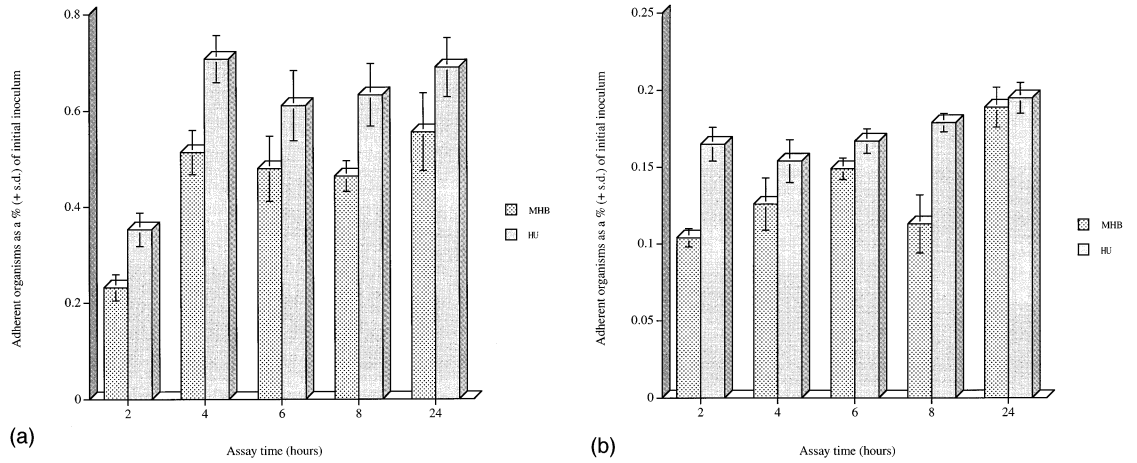

Fig. 1. Adherence of (a) *Enterococcus faecalis* and (b) *Escherichia coli* grown in Mueller-Hinton broth and human urine to polyurethane.

cal conditions (Denyer et al., 1990). The atmospheric growth conditions can affect the adherence properties of microorganisms. Wilcox et al. (1991) found that when 50 strains of coagulase-negative staphylococci were cultured in an atmosphere containing 5% CO_2, their mean adherence to silicone rubber and polystyrene was reduced by 86% and 84%, respectively, although occasional strains adhered in greater numbers to the surfaces after CO_2 atmospheric incubation. Wassall et al. (1994) showed that the adherence to glass of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was significantly greater when the organisms were grown in an atmosphere containing 5% CO_2.

Surface charge and hydrophobicity influence the initial attachment of a bacterium to a biomaterial surface. In this study, the hydrophobic characteristics of two isolates were determined using the BATH assay, which showed that the *Escherichia coli* isolate was hydrophilic and the *Enterococcus faecalis* isolate hydrophobic. Several other methods for assessment of hydrophobicity have also been reported including hydrophobic interaction chromatography (Hjerten et al., 1974) and the salt aggregation test (Lindahl et al., 1981). The BATH method has been shown to correlate well with other partitioning-based methods of cell surface hydrophobicity measurement such as hydrophobic interaction chromatography, although less so with the salt aggregation test (Jones et al., 1991).

The initial steps involved in bacterial adhesion to a biomaterial surface may be explained by colloid chemical theories such as the DLVO theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). According to this theory, the positioning of a bacterium relative to a surface is determined by attractive Van der Waals' forces and repulsive electrostatic forces. If the electrostatic forces are high the bacterium will be repelled by the surface and remain in suspension. However, as the distance between the bacterial cell and the surface is shortened, stronger adhesive forces begin to dominate, which are enhanced by extracellular polymers and bacterial cell structures. When the repulsive forces are overcome, bacterial adhesion to the surface takes place. The reduction in the surface charge of the two isolates when grown in human urine, as indicated by a reduction in their zeta potentials may, therefore, explain why adherence to polyurethane is enhanced when isolates are grown in human urine.

The process of conditioning film formation on a biomaterial surface will impact on bacterial adhesion to it. The contact data presented in this study indicate that the polyurethane surface chemistry had been dramatically altered by treat-

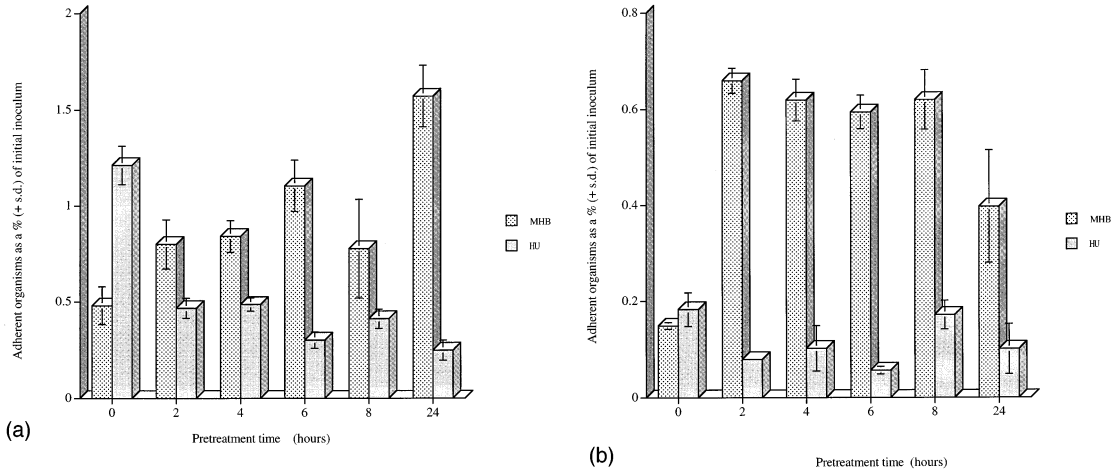

Fig. 2. Adherence of (a) *Enterococcus faecalis* and (b) *Escherichia coli* grown in Mueller-Hinton broth and human urine to polyurethane pretreated with human urine.

ment with human urine. Gorman et al. (1993) showed that when used and unused peritoneal catheters which had previously shown differences in bacterial adherence were pretreated with artificial spent peritoneal dialysate, no differences in adherence were apparent. The authors concluded that regardless of the underlying surface, a similar surface appeared to be presented to bacteria when long-dwell and control catheters became coated with protein.

Pretreatment of polyurethane with human urine in this study significantly increased the adherence of both isolates when grown in MHB. The increase in adherence was apparent after 2 h, indicating that a conditioning film is rapidly formed on the biomaterial surface. However, when the isolates were grown in human urine, polyurethane pretreatment with human urine significantly decreased their adherence. It appears that in this case, the deposition of a conditioning film on the biomaterial surface alters its surface properties, providing increased resistance to bacterial adherence. Growth of both isolates in human urine resulted in a reduction in their surface charge. Pretreatment of polyurethane with human urine alters the biomaterial surface properties which may render it more resistant to bacterial adherence. Weerkamp et al. (1988) have shown that coating dental surfaces with salivary proteins reduced bacterial adhesion, a phenomenon they attributed to an increased negative charge as a result of protein deposition.

Biomaterials will continue to have the associated problems of bacterial adhesion and infection. The examination of bacterial adhesion to surfaces in vitro is best carried out using clinically derived isolates, in physiological suspending media, as environmental factors impact on their adherence potential. In this study, *Enterococcus faecalis* and *Escherichia coli* displayed greater adherence to polyurethane when cultured in human urine. The deposition of host macromolecules onto the biomaterial surface is also a modifier of bacterial adhesion. Polyurethane treated with human urine had greater numbers of adherent MHB-cultured bacteria than untreated polyurethane, although conditioned polyurethane displayed reduced numbers of adherent human urine-cultured bacteria. Conditions closely resembling the in vivo situation should be used for in vitro bacterial adherence studies, especially if strategies for minimising bacterial adherence are under examination.

References

Bonner, M.C., Keane, P.F. and Gorman, S.P., Effect of growth conditions on the cell surface characteristics of

ureteral stent biofilm isolates and their adherence to polyurethane. *J. Pharm. Pharmacol.*, 47 (1995) 1095.

Carballo, J., Ferreiros, C.M. and Criado, M.T., Importance of experimental design in the evaluation of the influence of proteins in bacterial adherence to polymers. *Med. Microbiol. Immunol.*, 180 (1991) 149–155.

Denyer, S.P., Davies, M.C., Evans, J.A., Finch, R.G., Smith, D.G.E., Wilcox, M.H. and Williams, P., Influence of carbon dioxide on the surface characteristics and adherence potential of coagulase-negative staphylococci. *J. Clin. Microbiol.*, 28 (1990) 1813–1817.

Derjaguin, B.V. and Landau, V., Theory of the stability of strongly charged lyophobic sols and the adhesion of strongly charged particles in solutions of electrolytes. *Acta Physicochimica USSR*, 14 (1941) 633–622.

Gorman, S.P., Mawhinney, W.M., Adair, C.G. and Issouckis, M., Confocal scanning laser microscopy of CAPD catheter surface microrugosity in relation to recurrent peritonitis. *J. Med. Microbiol.*, (1993) 411–417.

Hjerten, S., Rosengren, J. and Pahlman, S., Hydrophobic interaction chromatography. The synthesis and use of some alkyl and aryl derivatives of agarose. *J. Chromatogr.*, 101 (1974) 281–288.

Jones, D.S., Gorman, S.P., McCafferty, D.F. and Woolfson, A.D., The effects of three non-antibiotic, antimicrobial agents on the surface hydrophobicity of certain micro-organisms evaluated by different methods. *J. Appl. Bacteriol.*, 71 (1991) 218–227.

Keane, P.F., Bonner, M.C., Johnston, S.R., Zafar, A. and Gorman, S.P., Characterisation of biofilm and encrustation on stents in vivo. *Br. J. Urol.*, 73 (1994) 687–691.

Lindahl, M., Faris, A., Wadstrom, T. and Hjerten, S., A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. *Biochim. Biophys. Acta*, 677 (1981) 471–476.

Reid, G., Beg, H.S., Preston, C.A.K. and Hawthorn, L.A., Effect of bacterial, urine and substratum surface tension properties on bacterial adhesion to biomaterials. *Biofouling*, 4 (1991) 171–176.

Reid, G., Tieszer, C., Foerch, R., Busscher, H.J., Khoury, A.E. and Vandermei, H.C., The binding of urinary components and uropathogens to a silicone latex urethral catheter. *Cells Mater.*, 2 (1992) 253–260.

Rosenberg, M., Gutnick, D. and Rosenberg, E., Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.*, 9 (1980) 29–33.

Tunney, M.M., Gorman, S.P. and Patrick, S., Infection associated with medical devices. *Rev. Med. Microbiol.*, 7 (1996), 195–205.

Verwey, E.J.W. and Overbeek, J.T.G. In *Theory of the Stability of Lyophobic Colloids*, Elsevier, Amsterdam, 1948.

Wassall, M.A., McGarvey, A. and Denyer, S.P., Influence of growth conditions on adherence potential and surface hydrophobicity of urinary tract isolate. *J. Pharm. Pharmacol.*, 46 (1994) 1044.

Weerkamp, A.H., Uyen, H.M. and Busscher, H.J., Effect of zeta potential and surface energy on bacterial adhesion to uncoated and saliva-coated human enamel and dentin. *J. Dent. Res.*, 67 (1988) 1483–1487.

Wilcox, M.H., Finch, R.G., Smith, D.G.E., Williams, P. and Denyer, S.P., *J. Antimicrob. Chemother.*, 27 (1991) 577–587.