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Standardisation and comparison of methods employed for microbial cell surface hydrophobicity and charge determination

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Abstract

Whilst there are a number of methods available to characterise the cell surface hydrophobicity (CSH) and cell surface charge (CSC) of microorganisms, there is still debate concerning the correlation of results between individual methods. In this study, the techniques of bacterial adherence to hydrocarbons (BATH) and hydrophobic interaction chromatography (HIC) were used to measure CSH. Electrostatic interaction chromatography (ESIC) and zeta potential (ZP) measurements were used to determine CSC. To allow meaningful comparisons between the BATH and HIC tests, between ESIC and ZP and also between CSH and CSC, the buffer systems employed in each test were standardised (phosphate buffered saline, pH 7.3, 0.01 mM). Isolates of *Staphylococcus epidermidis* derived from microbial biofilm were used as the test organism in this study. The isolates examined exhibited primarily medium to high CSH and a highly negative CSC. Good correlation of CSH measurement was observed between the BATH and HIC tests ($r = 0.89$). Good correlation was observed between ESIC (anionic exchange column) and ZP measurements. No correlations were observed between isolate CSC and either increased or decreased CSH. It is recommended that whenever comparisons of various methods to determine either CSC or CSH (by partitioning methods), the buffer systems should remain constant throughout to achieve consistency of results.

Keywords: Staphylococcus epidermidis; Hydrophobicity; Charge; BATH; HIC; ESIC; Correlation

1. Introduction

Adherence of microorganisms is accepted as the first (requisitory) step in the process of colonisation of inert surfaces and host epithelial cells (Busscher et al., 1990). The DLVO theory is often applied to describe microbial adherence. As two similarly charged bodies approach each other they are subjected to both attractive and repulsive forces which are additive in effect (Busscher et al., 1990; Fowler and Jones, 1992). Typically, at long distances of separation (circa 10 nm) attractive forces, including hydrophobic interactions between hydrophobic molecules on the surface of the bacterial cells and the inert surface, predomi-

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nate and serve to form a reversible attractive interaction. At shorter distances, an overlap of the adsorbed counter ion clouds, which account for the apparent cell surface charge (CSC), on each surface serves as a repulsion barrier between the two cells. Finally, irreversible adhesion between microbial cells and the inert substrate occurs via specific interactions between stereochemically complementary molecules on the surface of the microorganism and the inert substratum (Busscher et al., 1990; Fowler and Jones, 1992; Bunt et al., 1993). Hydrophobic interactions have been reported to contribute to adhesion processes involving bacteria and a range of inert surfaces, e.g., glass (Gilbert et al., 1991), polymers (Van Pelt et al., 1985; Klotz et al., 1989) and mineral surfaces (Stenstrom, 1989). However, some authors have reported poor correlation between cell surface hydrophobicity (CSH) and microbial adherence to inert surfaces (e.g., Hogt et al., 1985; Bandin et al., 1989). Similarly, the role of microbial surface charge in microbial adhesion processes is also unclear, with some reports advocating (e.g., Satou et al., 1988; Miyake et al., 1990) and others rejecting (e.g., Van Loosdrecht et al., 1987; Klotz, 1994) a primary role for this surface property. There are a range of methods used to determine CSH including microbial adherence to hydrocarbons (MATH), bacterial adherence to hydrocarbons (BATH), salt aggregation test (SAT) and contact angle measurements (CAM) (Gorman, 1991; Jones et al., 1991; Bunt et al., 1993). Microbial surface charge is often determined using electrostatic interaction chromatography (ESIC) or by measurement of the electrophoretic mobilities (and hence the zeta potential (ZP) of cells) (Busscher et al., 1990). The choice of method used to characterise microbial cell surface properties may contribute to the apparent discrepancies concerning the role of these surface properties in microbial adherence as the fundamental concepts in these tests may differ. In addition, variations in the experimental conditions employed by different research groups for any particular test may lead to further complications. Previously, we have shown that CSH determination using the BATH and hydrophobic interaction chromatography (HIC) methods is dependent on the buffer system employed (Bunt et al., 1993, 1995).

Therefore, the aim of this study was to examine the cell surface characteristics (CSH and CSC) of *Staphylococcus epidermidis* using the BATH, HIC, ESIC and ZP methods which have been standardised with respect to the composition of the buffer system of the aqueous phase. This will allow a meaningful comparison of the various methods in the absence of potential experimental interactive effects. The isolates employed in this study were obtained from microbial biofilm present on the surface of continuous ambulatory peritoneal dialysis Tenchkoff catheters (Gorman et al., 1994).

2. Materials and methods

2.1. Materials

Xylene, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate and sodium chloride were purchased from BDH (Poole, Dorset, UK).

Octyl-Sepharose CL-4B, Sephadex-CM and Sephadex-QAE were obtained from Pharmacia Fine Chemicals, UK.

All other chemicals were of AnalaR, or equivalent, quality.

2.2. Bacterial isolates

Isolates were recovered from microbial biofilm present on Tenckhoff catheters, identified and stored as previously described by us (Gorman et al., 1994). When required, stationary phase organisms were harvested by introduction of a sample of stored isolate into pre-warmed Mueller Hinton Broth (MHB, Oxoid) and incubating at 37°C in an orbital incubator for 16 h. Following this the bacterial cells were washed three times with, and finally suspended in sterile phosphate buffered saline (PBS, pH 7.3 0.01 mM) to the required cell count (10^9 colony forming units, cfu mL^{-1}).

2.3. Determination of bacterial cell surface hydrophobicity (CSH)

Two methods were employed to determine CSH of the bacterial isolates, namely, the BATH test

(Jones et al., 1991; Bunt et al., 1993) and HIC (Jones et al., 1991; Bunt et al., 1995). In the BATH test, an aliquot (4.8 ml) of a bacterial suspension in PBS was added to xylene (0.4 ml) and vortex mixed at constant speed for 5 min. Following equilibration, the lower aqueous layer was carefully removed and its absorbance measured at 400 nm. The hydrophobicity is expressed as the percentage of the applied cell suspension absorbance which had been excluded from the aqueous phase.

In HIC, a volume (1 ml) of a bacterial suspension in PBS buffer was added to a hydrophobic (octyl-sepharose CL-4B) column and flushed through with an excess of PBS buffer. The eluent was collected and after equilibration the absorbance determined at 400 nm. hydrophobicity, i.e., the percentage of organisms retained on the column, was calculated as a percentage of the applied cell suspension absorbance.

For both the BATH test and HIC all determinations were performed in quadruplicate and the standard deviations of replicates were always within 5% of the mean.

2.4. Determination of cell surface charge

ESIC was employed to determine microbial CSC using a modification of the technique described by Onaolapo et al. (1987). The bacterial suspension (circa 10^9 cfu mL^{-1}, stationary phase, 1 ml) was added to either a sephadex-CM (cation-exchange) or sephadex-QAE (anion-exchange) column and flushed through with an excess volume of PBS. The eluent was collected after equilibration, the absorbance determined at 400 nm and the percentage of organisms retained on both column types was calculated as a percentage of the applied cell suspension absorbance. All experiments were performed in quadruplicate and the results are expressed as mean values.

ZP of bacterial suspensions (10^9 cells mL^{-1}) were determined using a Malvern Zetasizer IV (Malvern Instruments, Malvern, UK). Aliquots of bacterial suspensions were placed into a ZET 5104 capillary cell at 25°C and ZP measurements performed at least six times for individual samples (field strength 10–20 V cm^{-1}, electrode spacing 50 mm, dielectric constant 78.54). The ZPs of at least three individual replicate samples were determined.

In all cases, coefficient of variations of each experiment never exceeded 5%.

3. Results and discussion

Employing the CSH classification scheme described by Martin et al. (1989) and Schneider and Riley (1991), isolates with a % hydrophobicity greater than 70% were classified as highly hydrophobic and those with a hydrophobicity index less than 30% were classified as highly hydrophilic. The cell surface characteristics of the isolates of *Staphylococcus epidermidis* under examination are presented in Table 1. The isolates exhibited a variety of CSH, ranging from 27.47–81.25% and from 45.43–96.00% when evaluated by the BATH and HIC techniques, respectively. Using the BATH method 33.3% of isolates were classified as highly hydrophilic, 41.7% displayed medium hydrophobicity and 25.0% of isolates were highly hydrophobic. In comparison, 0.0%, 50.0% and 50.0% of isolates exhibited low, medium and highly hydrophobic CSH as determined by HIC. In addition, a good correlation ($r = 0.88$) between the CSH determined using the BATH test and HIC was observed in this study and is illustrated in Fig. 1.

All isolates exhibited an overall negative cell surface charge as demonstrated by the large percentage retention of isolates on the QAE-sephadex (anion-exchange) column and negative ZP and significantly lower percentage retention with the cation-exchange CM-sephadex column. CSC determination using ESIC (QAE-sephadex) correlated well with ZP measurements ($r > 0.9$). There was no evident correlation between isolate CSC (determined using either ESIC or ZP measurements) and either increased or decreased CSH as evaluated using HIC or BATH methods.

There is a lack of concensus concerning the exact contribution of CSH to microbial adherence to inert surfaces. Similarly, there is a controversy about the role of cell surface charge in the process of microbial adherence to inert surfaces (e.g., Van

Table 1
CSH and CSC of isolates of *Staphylococcus epidermidis* as determined by BATH test, HIC, ESIC and ZP measurements

Mean Cell surface properties					
Isolate Number	BATH (%)	HIC (%)	ESIC [a] (CM, %)	ESIC [b] (QAE, %)	ZP (−mV)
1	68.48	90.53	57.14	94.28	−38.20
2	81.25	96.00	54.10	88.78	−24.30
3	22.44	45.43	17.81	88.28	−24.30
4	27.27	48.71	67.91	100.00	ND
5	70.13	93.54	15.48	100.00	ND
6	34.07	48.97	53.52	100.00	ND
7	29.07	49.01	68.39	100.00	ND
8	78.40	70.37	79.21	100.00	−42.80
9	65.60	65.15	54.55	100.00	−37.60
10	52.95	70.70	64.01	99.18	ND
11	51.98	55.88	46.01	100.00	−44.40
12	73.61	92.10	69.36	100.00	ND

ND: Not Determined
[a] Cation-exchange column, see Materials and methods
[b] Anion-exchange column, see Material and methods

Loosdrecht et al., 1987; Onaolapo et al., 1987; Busscher et al., 1990). These conflicting opinions may be due, at least in part, to the methods employed to measure cell surface properties as these may not necessarily provide significant inter-method correlations. For example, a bacterium with a hydrophilic outer surface which also possesses fimbriae with hydrophobic binding properties may be characterised as hydrophobic by BATH, MATH and HIC, but hydrophilic by CAM (Rosenberg and Kjelleberg, 1986). In addition, bacteria which exhibit auto-aggregation properties will give a disproportionately high CSH when determined using SAT. Lack of inter-method correlation has also been suggested to be due to the inconsistency of experimental conditions from one test to another which in turn alters the final assessment of CSH (Bunt et al., 1993; Bunt et al., 1995). There have been a variety of buffers employed in the BATH test and HIC including phosphate-urea-magnesium (PUM) (Rosenberg et al., 1980), sodium chloride over a range of pH values (Mozes and Rouxhet, 1987; Allison et al., 1990), PBS (Minagi et al., 1986), and McIlvaine buffer (Bunt et al., 1993). Previously we have reported that microbial CSH assessment using BATH and HIC is dependent on both the ionic strength and ionic valence of the buffer system employed as the aqueous phase. Typically, increased ionic strength in the aqueous phase

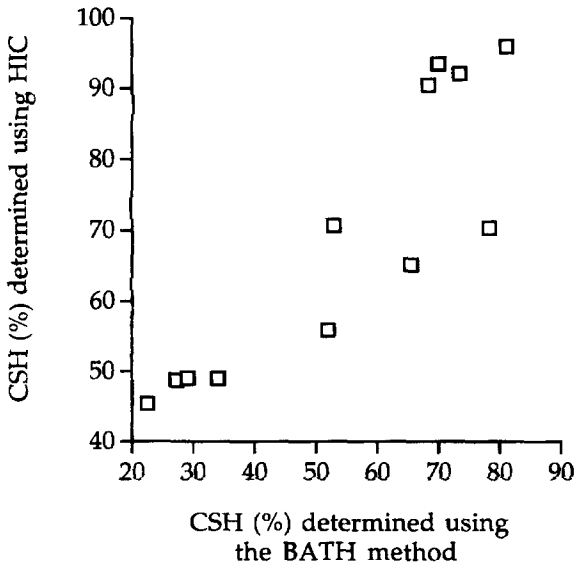

Fig. 1. Correlation between the bacterial adherence to hydrocarbons and hydrophobic interaction chromatography methods in the measurement of the cell surface hydrophobicity of isolates of *Staphylococcus epidermidis* from biofilm on Tenckhoff catheters.

increased CSH, as examined using BATH and HIC, whereas increasing the valence of the metal cation components of the buffer system increased and decreased the CSH when determined using HIC and BATH, respectively (Bunt et al., 1993, 1995). Therefore, failure to control these parameters may result in the erroneous characterisation of CSH and a lack of correlation between the two methods. Consequently, in the current study PBS at constant ionic strength was used in all measurements of cell surface properties. The resulting good correlation between the BATH method and HIC in the determination of CSH may be due to similarities in the underlying mechanism measuring the relative distribution between an aqueous and a non-aqueous phase. BATH and HIC are routinely employed to evaluate CSH, however, these methods have been shown in previous studies to exhibit a range of inter-method correlations, with some authors reporting good correlation (e.g., Klotz et al., 1985; Darnell et al., 1987) whereas others have reported moderate or organism dependent correlations (Schiemann and Swanz, 1985; Mozes and Rouxhet, 1987; Jones et al., 1991). However, Dillon et al. (1986) and Van der Mei et al. (1987) reported poor correlation between the BATH and HIC techniques in the determination of CSH. Interestingly, in the vast majority of studies which have compared BATH and HIC methods for the determination of CSH the authors have employed different buffer systems (of different ionic strengths) for each CSH test, which will consequently affect the overall assessment of CSH. Therefore, those studies which have reported correlations (high or low) may have unwittingly biased the outcome of the statistical analysis due to these differences in the buffer compositions in each method. Hence, this highlights the need for standardised methods when comparing different methods for the determination of CSH.

Most bacteria possess a negative cell surface charge under normal environmental conditions (Rosenberg and Doyle, 1990). As expected, in this study all isolates exhibited a highly negative cell surface. Interestingly, some isolates exhibited a marked interaction with the cationic-exchange column. This may be possibly interpreted as the presence of cationic molecules on the cell surface, although, these are outnumbered by their negatively charged counterparts. Once again, although ESIC has been employed by several authors to examine CSC, there have been extensive variations in the experimental conditions used, e.g., type and mass of chromatographic column and buffer system (Pedersen, 1980; Hogt et al., 1985; Onaolapo et al., 1987). Variations in these experimental factors will alter the interaction of the bacteria with the column which will therefore affect the assessment of surface charge by ESIC. Similarly, alterations in the buffer systems will alter the final assessment of surface charge as determined by ZP measurement. The positive correlation between CSC and ZP of the isolates observed is most likely due to the standardised buffer system employed. It is anticipated that an inverse correlation should exist between surface charge and CSH as increased cell charge allows greater chances of polar interactions with an aqueous environment (Busscher et al., 1990). However, whilst some studies have shown that by decreasing negative CSC an increased CSH ensues (Miller and Ahearn, 1987; Goldberg et al., 1990), there have been other studies which have reported no correlation between CSC and CSH (Eisen and Reid, 1989; Ferreiros et al., 1989). The lack of correlation observed in this study may reflect the relative insensitivity of the CSH assessment techniques to detect changes in CSH resultant from altered CSC, as proposed by Weerkamp et al. (1988). Due to the standardisation of methods employed in this study, the possibilities of variations in experimental conditions accounting for this lack of correlation may be disregarded.

In conclusion, this study has shown that, by standardisation of buffer system composition, good correlations between the BATH test and HIC and between ESIC and ZP determinations were observed in the estimation of the CSH and CSC of isolates of *Staphylococcus epidermidis*, respectively. It is suggested that whenever more than one method is employed to determine either CSH or CSC, careful control of buffer parameters should be considered to ensure reproducibility between individual methods.

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