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In vitro and *in vivo* microbial adhesion and growth on argon plasma-treated silicone rubber voice prostheses

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Patients who undergo a total laryngectomy usually receive a silicone rubber voice prosthesis for voice rehabilitation. Unfortunately, biofilm formation on the esophageal side of voice prostheses limits their lifetime to 3–4 mon on average. The effects of repeated argon plasma treatment of medical grade, hydrophobic silicone rubber on *in vitro* adhesion and growth of bacteria and yeasts isolated from voice prostheses, as well as *in vivo* biofilm formation are presented here. *In vitro* experiments demonstrated that initial microbial adhesion over a 4 h time span to plasma-treated, hydrophilized, silicone rubber was generally less than on original, hydrophobic silicone rubber, both in the absence and presence of a salivary conditioning film on the biomaterial. Growth studies over a time period of 14 d at 37 °C in a modified Robbins device, showed that fewer *Candida* cells adhered on plasma-treated, hydrophilized silicone rubber as compared to on original, hydrophobic silicone rubber. For the *in vivo* evaluation of biofilm formation on plasma-treated silicone rubber voice prostheses, seven laryngectomized patients received a partly hydrophilized ''Groningen Button'' voice prosthesis for a planned evaluation period of 4 wk. After removal of the voice prostheses, the border between the hydrophilized and the original, hydrophobic side of the prostheses was clearly visible. However, biofilm formation was, unexpectedly, less on the original, hydrophobic sides, although the microbial compositions of the biofilms on both sides were not significantly different. Summarizing, this study demonstrates that *in vitro* microbial adhesion and growth on silicone rubber can be reduced by plasma treatment, but *in vivo* biofilm formation on silicone rubber voice prostheses is oppositely enhanced by hydrophilizing the silicone rubber surface. Nevertheless, from the results of this study the important conclusion can be drawn that *in vivo* biofilm formation on voice prostheses is controlled by the hydrophobicity of the biomaterials surface used.

1. Introduction

Total laryngectomy is a surgical treatment for extensive cancer of the larynx or hypopharynx. It entails surgical excision of the whole larynx including the vocal folds. Postoperatively, the respiratory tract and upper digestive tract are totally separated, with a permanent tracheostoma in the neck as inlet and outlet of the respiratory tract. The anatomy of a patient after laryngectomy with a voice prosthesis is shown in Fig. 1. Acquisition of voice and intelligible speech following this procedure is considered the main determinant of the quality of life. During the last decade, surgical voice restoration procedures comprising tracheo-oesophageal puncture techniques with insertion of a so-called voice prosthesis have greatly improved successful voice acquisition following laryngectomy [1–11]. These voice prostheses consist of a valve situated in the surgically created tracheo-oesophageal shunt which allows passage of air from the respiratory tract to the pharynx and the mouth, but prevents contents to pass from the digestive tract into the respiratory tract.

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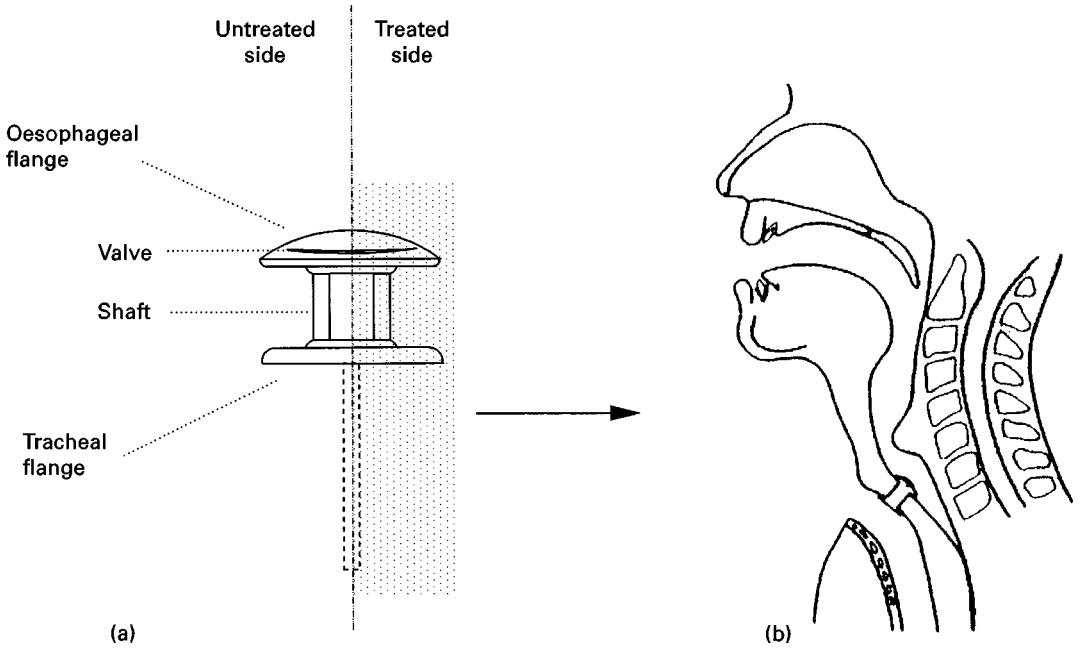

Figure 1 (a) Diagram of the "Groningen Button" voice prosthesis and (b) anatomy of the head–neck area after a total laryngectomy with a "Groningen Button" voice prosthesis placed into the tracheo-oesophageal shunt. Note that voice prostheses used for the evaluation of biofilm formation *in vivo* were only partly treated by argon plasma, as indicated in the figure.

Speech is produced by closing the stoma with a finger and forcing air through the valve to the upper digestive tract, where remaining muscular structures at the oesophageal entrance function as an alternative sound source. There are several types of voice prostheses available, such as the Groningen Button [3, 4], Herrmann-ESKA [5], Provox [6, 7], Traissac [8], Staffieri [9], Algaba [10] and Blom-Singer [11] voice prostheses. Most voice prostheses are made of silicone rubber because of its excellent mechanical and moulding properties.

Voice prostheses are placed in a non-sterile, humid, nutrient-rich environment and therefore become quickly colonized by micro-organisms [12–17]. Clinically, indwelling voice prostheses are replaced, on average, every 4 months [18] when, due to biofilm formation, patients complain about leakage of food and liquid or increased air-flow resistance [17]. Analysis of the biofilms on voice prostheses removed from patients demonstrated that the initially colonizing yeast strains were mainly *Candida albicans* and that *Candida tropicalis* was probably the predominant yeast strain in mature biofilms on voice prostheses [12–14]. Bacterial strains identified were of oral origin and included *Streptococcus mitis*, *Streptococcus sobrinus* and *Streptococcus salivarius* or were commensals from the skin, such as *Staphylococcus epidermidis* and other staphylococcal isolates [15].

Prevention of colonization was partly achieved *in vivo* using antifungal amphotericin B lozenges (10 mg four times daily), which significantly prolonged the life time and therewith reduced the air-flow resistance of the voice prostheses [19]. Prolonged administration of antifungal agents to patients is, however, uneconomical, undesirable because of the danger to induce resistance and the compliance of long-term medication is low. Therefore, it would be much more effective if deterioration of the prostheses by micro-organisms could be prevented by changing the material or its surface characteristics.

The formation of a biofilm is usually considered to start with the adsorption of conditioning film components [20], transport and adhesion of micro-organisms, attachment and growth, possibly followed by ingrowth of selected organisms, most notably *C. tropicalis* and *C. albicans* in the case of silicone rubber voice prostheses. Adsorption of conditioning film components and microbial adhesion to surfaces can be excellently studied *in vitro* using flow devices, such as a parallel plate flow chamber. Especially when combined with *in situ* observation methods and image analysis software, these systems have proved to be most versatile to study initial microbial adhesion phenomena [21]. Recently, also the biodeterioration of silicone rubber by ingrowing yeasts has been simulated *in vitro* using a modified Robbins device by passing yeasts, adhering to silicone rubber, through a cycle of nutritional feast and famine [16].

Microbial adhesion to surfaces is an interplay of the hydrophobicity and charge properties of the interacting surfaces [22]. Therefore, modification of the surface properties of silicone rubber by, for example, radio frequency plasma treatment, might reduce the adhesion and ingrowth of micro-organisms to voice prostheses and therewith improve their life time. Plasma treatment is often a useful method to hydrophilize polymer surfaces, but effects of a single plasma treatment of silicone rubber are usually transient due to, amongst other reasons, the high mobility of the siloxane backbone. However, repeated plasma treatments of silicone rubber yields more permanent effects [23]. Moreover, the hydrophilicity thus created on to silicone rubber surfaces can be preserved for several months by storing

the plasma-treated surfaces in a hydrophilic medium such as water [24].

The aim of this study was firstly to determine the influence of repeated argon plasma treatments of silicone rubber on initial microbial adhesion and yeast growth *in vitro*. To this end, adhesion of bacteria and yeasts to silicone rubber was studied in a parallel plate flow chamber, with and without an adsorbed salivary conditioning film. In addition, growth of yeasts adhering to silicone rubber was studied in a modified Robbins device. As a secondary aim, the clinical performance of argon plasma-treated voice prostheses was evaluated.

2. Materials and methods

2.1. Strains and growth conditions

The bacterial and yeast strains used in this study were isolated from "Groningen Button" voice prostheses [15], removed from patients complaining either about leakage or blocking of the valve and included two bacterial strains *Streptococcus salivarius* GB 24/9, cultured in Todd Hewitt broth, *Staphylococcus epidermidis* GB 9/6, cultured in Tryptone Soya broth and the two yeast strains (*Candida albicans* GB 1/2 and *Candida tropicalis* GB 9/9) cultured in Brain Heart Infusion broth, Culture media were purchased from Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK. All micro-organisms were inoculated from agar plates into a batch culture for 24 h at 37 °C and ambient air, which was used to inoculate a second culture which was grown for 16 h under similar conditions.

The micro-organisms were harvested by centrifugation (5 min at 4000 *g* for bacterial and 10 000 *g* for the yeast strains), washed twice with Millipore® water and resuspended in adhesion buffer (50 mM KCl, 2 mM potassium phosphate and 1 mM $CaCl_2$, pH 6.8), bacteria to a concentration of 3×10^8 per ml and yeasts to a concentration of 3×10^6 per ml, as determined in a Bürker–Türk counting chamber.

2.2. Saliva coating

From healthy volunteers of both sexes, human whole saliva was collected into ice-chilled cups. Saliva was stimulated by the volunteers chewing Parafilm®. After the saliva was pooled and centrifuged at 10 000 *g* for 10 min at 4 °C, phenylmethylsulphonylfuoride (0.2 M) was added to a final concentration of 1 mM as a protease inhibitor. The solution was again centrifuged, dialysed for 48 h at 4 °C against Millipore® water and freeze-dried for storage. A solution of 1.5 mg ml^{-1} freeze-dried stock in adhesion buffer will be denoted as (reconstituted human whole) saliva.

2.3. Silicone rubber and voice prostheses

Silastic®/Medical Grade Silicone Rubber, a dimethyl and methylvinyl siloxane copolymer, (Q7-4750, Dow Corning) kit was purchased. Plates 0.5 mm thick 50 mm × 76 mm (for flow chamber studies) or discs 1 mm thick and 6.3 mm diameter (for studies in the modified Robbins device) were produced following the procedures suggested by the manufacturer. Briefly, equal proportions of parts A and B were thoroughly blended together and injected into a mould at room temperature. Subsequently, the silicone rubber was immediately cured at 200 °C for 50 min. Finally, samples were cleaned in a 2% RBS 35 (Omnilabo International B.V., Breda, The Netherlands) detergent solution under simultaneous sonication (5 min, 150 W) and thoroughly rinsed in Millipore® grade water and absolute ethanol (> 96%).

The "Groningen Button Ultra Low Resistance" voice prostheses were obtained from Medin Instruments and Supplies (Groningen, The Netherlands) and plasma-treated as described below.

2.4. Repeated plasma treatment of silicone rubber

The silicone rubber plates (for use in the flow chamber), discs (for use in the modified Robbins device) and voice prostheses were repeatedly (six times at 24 h time intervals) glow discharge treated in a d.c. modified Edwards sputter coater S150B with a cylindrical reaction chamber (inner diameter 15 cm, height 11 cm, electrode diameter 10 cm with a separation distance of 4.5 cm). All plasma treatments were done under 0.2 mbar argon pressure, at a power of 5 W for 5 min. Argon gas (99.996%) was obtained from Hoekloos Nederland B.V., Groningen, The Netherlands. Samples were stored in ambient air in between plasma treatment. After the last argon plasma treatment, treated samples were used immediately for parallel plate flow chamber or modified Robbins device experiments, while treated voice prostheses were stored in sterile water prior to placement in a patient. Treated voice prostheses were never stored for longer than 3 mon.

The silicone rubber discs for use in the modified Robbins device as well as the "Groningen Button" voice prostheses (see Fig. 1) were partly exposed to the argon plasma for only *half* of their surfaces by placing the samples in a plaster cast (Dental superhart gypsum, New Fujirock, GC Corporation, Tokyo, Japan), during treatments [25]. Therewith, the growth experiments in the Robbins device as well as the clinical evaluation of the surface modification *in vivo*, could be done on one disc or voice prosthesis, respectively.

Water contact angles were measured on each half of these samples, to ensure that the silicone rubber hydrophobicity had been maintained and that the plasma had created a hydrophilic surface. Typically, the advancing water contact angles on the hydrophobic side of a silicone rubber disc or voice prosthesis were 115°, while the hydrophilized sides had advancing water contact angles of 15°.

2.5. The parallel plate flow chamber and image analysis

The flow chamber and image analysis system have been previously described [21]. Images were taken from the bottom plate of the parallel plate flow chamber which consisted of a silicone rubber or plasma-treated silicone rubber sample affixed to

a thicker (1.5 mm) perspex plate. The top plate of the chamber was made of glass.

Deposition was observed with a CCD-MXRi camera (High Technology, Eindhoven, The Netherlands) mounted on a phase contrast microscope (Olympus BH-2) equipped with a $40\times$ ultra long working distance objective (Olympus ULWD-CD Plan 40 PL) for experiments with bacteria and with a $10\times$ objective for experiments with yeasts. The camera was coupled to an image analyser (TEA, Difa, Breda, The Netherlands). Each live image (512×512 pixels with 8 bits resolution), obtained after summation of eight consecutive images (time interval 1 s) in order to enhance the signal-to-noise ratio and to eliminate moving micro-organisms from the analysis. Subsequently, adhering micro-organisms were discriminated from the background by a single grey-value threshold yielding a binary black and white image and the number of adhering micro-organisms was counted. An image covers a surface area of 0.017 mm^2 at the magnification used for bacterial experiments and 0.3 mm^2 at the magnification employed in the experiments with yeasts.

Prior to each experiment, all tubes and the flow chamber were filled with adhesion buffer, while care was taken to remove air bubbles from the system. Flasks, containing microbial suspension, buffer and saliva when appropriate, were positioned at the same height with respect to the chamber to ensure that immediately after the flows were started, all fluids would circulate through the chamber at the desired shear rate of $10\,s^{-1}$ ($0.025\,ml\,s^{-1}$), which yields a laminar flow (Reynolds number 0.6).

When a salivary conditioning film was required, flow was switched first to saliva for 1.5 h, followed by a flow of buffer during 1 h to remove all remnants of saliva from the tubes and chamber. The microbial suspension was circulated through the system for 4 h and images were obtained at the highest possible rate. The initial increase in the number of adhering micro-organisms with time was expressed in a so-called initial deposition rate, j_0 ($cm^{-2}\,s^{-1}$), i.e. the number of micro-organisms adhering per unit time and area. The number of micro-organisms adhering after 4 h, n_{4h}, was taken as an estimate of microbial adhesion in a more advanced state of the process. Finally, while focus was maintained on the same spot of the substratum, the number of adhering micro-organisms in this field of view was compared with the number of micro-organisms that remained adhering after passing an air bubble through the chamber to obtain an indication of the adhesive forces [26]. All values given in this paper are the means of experiments on at least two separately prepared samples.

2.6. Modified Robbins device

A modified Robbins device [27], in which ten silicone rubber discs could be inserted simultaneously has been used. Silicone rubber discs used in the Robbins device were exposed for only half of their surface to the plasma treatment. First, the device was inoculated with an overnight culture of the appropriate yeast strain, either *C. albicans* GB 1/2 or *C. tropicalis* GB 9/9. The device was then perfused for 7 d with defined growth medium (glucose $7.5\,g.l^{-1}$, $(NH_4)_2SO_4$) $3.5\,g.l^{-1}$, *L*-asparagine $1.5\,g.l^{-1}$, *L*-histidine $10\,mg.l^{-1}$, DL-methionine $20\,mg.l^{-1}$, DL-tryptophane $20\,mg.l^{-1}$, KH_2PO_4 $1\,g.l^{-1}$, $MgSO_4.\,7H_2O$ $500\,mg.l^{-1}$, NaCl $500\,mg.l^{-1}$, $CaCl_2\cdot2H_2O$ $500\,mg.l^{-1}$, yeast extract $100\,mg.l^{-1}$, H_3BO_3 $500\,\mu g.l^{-1}$, $ZnSO_4.\,7H_2O$ $400\,\mu g.l^{-1}$, Fe(III)Cl_3 $120\,\mu g.l^{-1}$, $Na_2MoO_4.\,2H_2O$ $200\,\mu g.l^{-1}$, KI $100\,\mu g.l^{-1}$, $CuSO_4 5H_2O$ $40\,\mu g.l^{-1}$) and subsequently, also for 7 d, with phosphate buffered saline (10 mM potassium phosphate and 150 mM NaCl, pH 7.0), in order to mimic the varying availability of nutrients occurring *in vivo*. The discs were removed after 14 d for scanning electron microscopy (SEM). The temperature of the device was maintained at 35–37 °C during all experiments.

For electron microscopy, the discs were first flushed with 6.8% sucrose and 0.2% ruthenium red in 0.1 M cacodylate buffer (pH 7.4). After fixation in 2% glutardi-aldehyde and 0.1 M cacodylate buffer, for 2–24 h at 4 °C, discs were flushed a second time. Post-fixation was carried out in 1% OsO_4 and 0.2% ruthenium red in 0.1 M cacodylate buffer by gently shaking for 3 h at room temperature. This procedure removed most of the thick biofilm present on the discs, leaving only those cells that were in direct contact with the silicone rubber and that potentially showed ingrowth.

Dehydration involved sucrose and 0.1 M cacodylate buffer: 20 min; double distilled water: 3×10 min; ethanol series, 30%, 50% and 70%: each 20 min; ethanol 100%: 4×30 min. After critical-point drying with CO_2 for 4 h, the specimens were mounted on SEM stubs and sputter-coated with gold and palladium (15 nm).

2.7. Clinical evaluation of plasma-treated voice prostheses

Seven laryngectomized patients with at least 6 mon experience wearing a voice prosthesis, received a partly plasma-treated Groningen Button voice prosthesis for a planned evaluation period of approximately 4 wk [25]. After removal from the patients, biofilm formation on the modified and unmodified side of the prosthesis were compared by visual and scanning electron microscopical evaluation, the latter as described for the sample discs in the Robbins device experiments. Visual evaluation included a planimetric evaluation of the percentage area of the oesophageal flange covered by biofilm.

Furthermore, microbial compositions of the biofilms on modified and unmodified sides of the oesophageal valves of the prostheses were compared by plating on Brain Heart Infusion and blood agar plates at 37 °C under aerobic conditions [14]. To this end, micro-organisms were separately isolated from both the plasma-treated and untreated side of the prostheses, transfered to 4.5 ml reduced transport fluid (RTF) and sonicated for 20 s. Subsequently, the microbial suspension was diluted up to six times in RTF and from each dilution micro-organisms were grown on Brain Heart Infusion and blood agar plates at 37 °C in ambient air. Isolated bacterial colonies were first

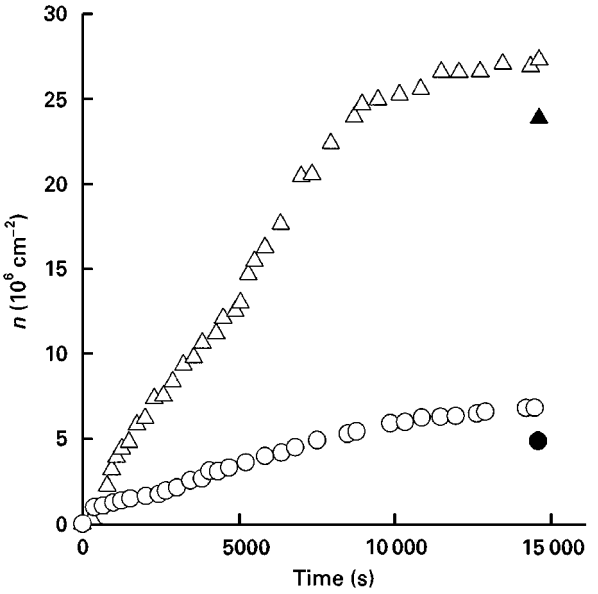

Figure 2 Adhesion kinetics of *Staph. epidermidis* GB 9/6 to (○) repeatedly argon plasma-treated, hydrophilized and (△) original, hydrophobic silicone rubber (○). Closed symbols denote the number of adhering bacteria after passing an air bubble through the flow chamber.

examined by light microscopy and Gram-staining. Then, the bacterial strains were identified by the Biolog system and the yeast strains by API ID 32C test system (BioMérieux).

3. Results

3.1. *In vitro* flow-chamber experiments

Fig. 2 presents an example of the adhesion kinetics for a microbial strain to argon plasma-treated, hydrophilized and original, hydrophobic silicone rubber. Quantitative characteristics of these plots, including the initial deposition rate, j_0, the adhesion in a stationary end-point, n_{4h}, and the percentage of adhering micro-organisms detached after the passage of an air bubble through the flow chamber, are shown in Figs 3 and 4 for the bacterial and yeasts strains, respectively. As can be seen from Figs 3 and 4, initial deposition rates to plasma-treated, hydrophilized silicone rubber were generally lower than to hydrophobic silicone rubber, both in the absence and presence of a salivary conditioning film. Similarly, also the numbers of adhering micro-organisms after 4 h were lower for plasma treated, hydrophilized than for original, hydrophobic silicone rubber surfaces. In addition, some strains were more readily detached by the passage of an air bubble from plasma treated, hydrophilized silicone rubber.

Although the above summarizing statements are not all equally valid for all strains tested here, the *in vitro* flow chamber studies appear to point out that argon plasma treatment could be a promising method to discourage biofilm formation on silicone rubber voice prostheses.

3.2. *In vitro* Robbins device experiments

All discs removed from the Robbins device were covered by a thick biofilm, as could be seen with the naked eye, but during preparation for electron microscopy most of the biofilm detached, leaving only the micro-organisms in direct contact with the silicone rubber surface.

Fig. 5 shows scanning electron micrographs of argon plasma-treated, hydrophilized and original, hydrophobic sides of the silicone rubber discs after growth of *C. tropicals* GB 9/9 and *C. albicans* GB 1/2, respectively, under dynamic nutrient conditions at 37 °C. In line with the flow-chamber studies, involving only adhesion and not growth, fewer *Candida* cells were found adhering on the hydrophilized side as compared to the original hydrophobic silicone rubber. No ingrowth of the adhering yeasts was observed.

3.3. Evaluation of *in vivo* biofilm formation on voice prostheses

In Fig. 6, it can be seen that the border between the plasma-treated, hydrophilized and the original, hydrophobic side of the oesophageal flange of the voice prostheses removed from laryngectomized patients is clearly visible from differential biofilm formation, which is significantly less on the original, hydrophobic side (Fig. 6a). Quantitative support for this statement can be found in Table I, summarizing the number of colony-forming units (CFU) and planimetrically scored biofilm formation on both sides of the voice prostheses as removed from the patients. In all patients, hydrophilizing the silicone rubber enhanced biofilm formation. Ingrowth of adhering bacteria and yeasts strains was also seen, especially on the hydrophilized side, as shown in Fig. 6b, yielding crater-like defects surrounded by circular deformations (see Fig. 6c).

Table II compares the microbial compositions of the biofilms on each side of the oesophageal flange of prostheses removed from patients. Five different *Candida* species and 16 different bacterial strains were isolated. Among the *Candida* species isolated, no *C. tropicalis* strains were found, while the bacterial strains identified included commonly isolated strains and species like lactobacilli, staphylococci and streptococci. However, in general the plasma-treated,

TABLE I The number of colony forming units per unit area (CFU) and planimetric biofilm scores on partly hydrophilized "Groningen Button" silicone rubber voice prostheses removed from laryngectomized patients, after a planned evaluation period of 4 wk

	Original, hydrophobic side		Hydrophilized side	
Patient	Planimetric biofilm score (%)	CFU (10^6 cm^{-2})	Planimetric biofilm score (%)	CFU (10^6 cm^{-2})
A	30	0.2	90	8.6
B	10	3.0	100	56.2
C	20	0.1	30	38.7
D	10	3.3	70	41.1
E	20	–	40	–
F	5	0.2	30	1.6
G	30	3.9	90	117

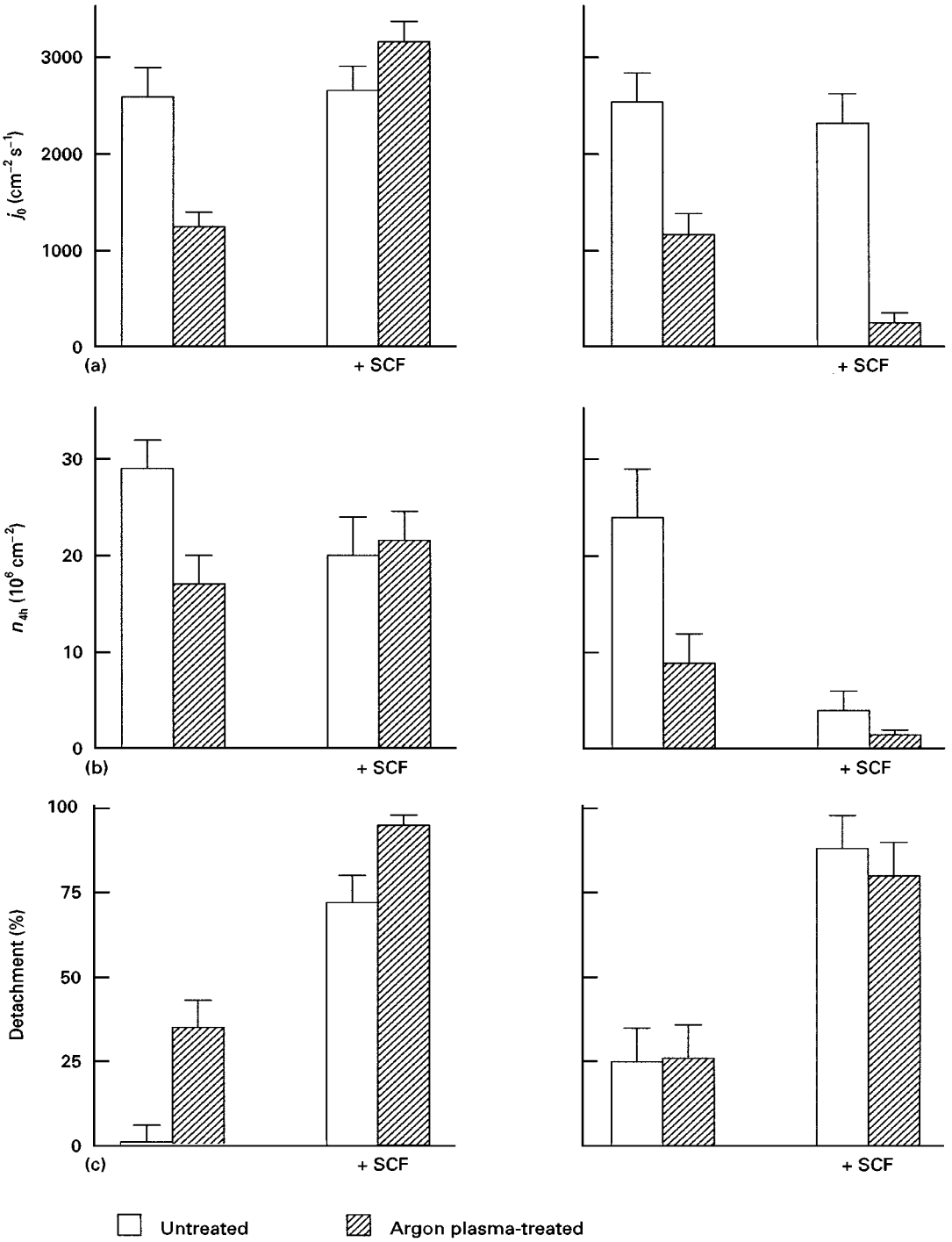

Figure 3 (a) Initial deposition rates, j_0, (b) numbers of adhering bacteria after 4 h n_{4h} and (c) percentages of bacteria detached after passing an air bubble through the flow chamber to argon plasma-treated hydrophilized and original, hydrophobic silicone rubber in the absence and presence of a salivary conditioning film (SCF), for two bacterial strains; *Strept. salivarius* GB 24/9 (left-hand panel) and *Staph. epidermidis* GB 9/6 (right hand panel).

hydrophilized side of the valves did not attract a significant number of different microbial strains and species as compared to the original, hydrophobic side.

4. Discussion

In this paper we describe the *in vitro* and *in vivo* evaluation of the fouling properties of argon plasma-treated, hydrophilized silicone rubber voice prostheses in laryngectomized patients. The evaluation therewith includes all steps in the development of a biofilm, ranging from adsorption of conditioning film components and initial adhesion of yeasts and bacteria, growth, possibly followed by ingrowth, as well as long-term *in vivo* biofilm formation. Most interestingly, *in vitro* experiments involving two bacterial and two yeast strains isolated from voice prostheses indicated that hydrophilized silicone rubber might perform better *in vivo* than original, hydrophobic silicone rubber. Although *in vivo* experiments proved the opposite, evaluation of biofilm formation on partly hydrophilized voice prostheses most decisively showed

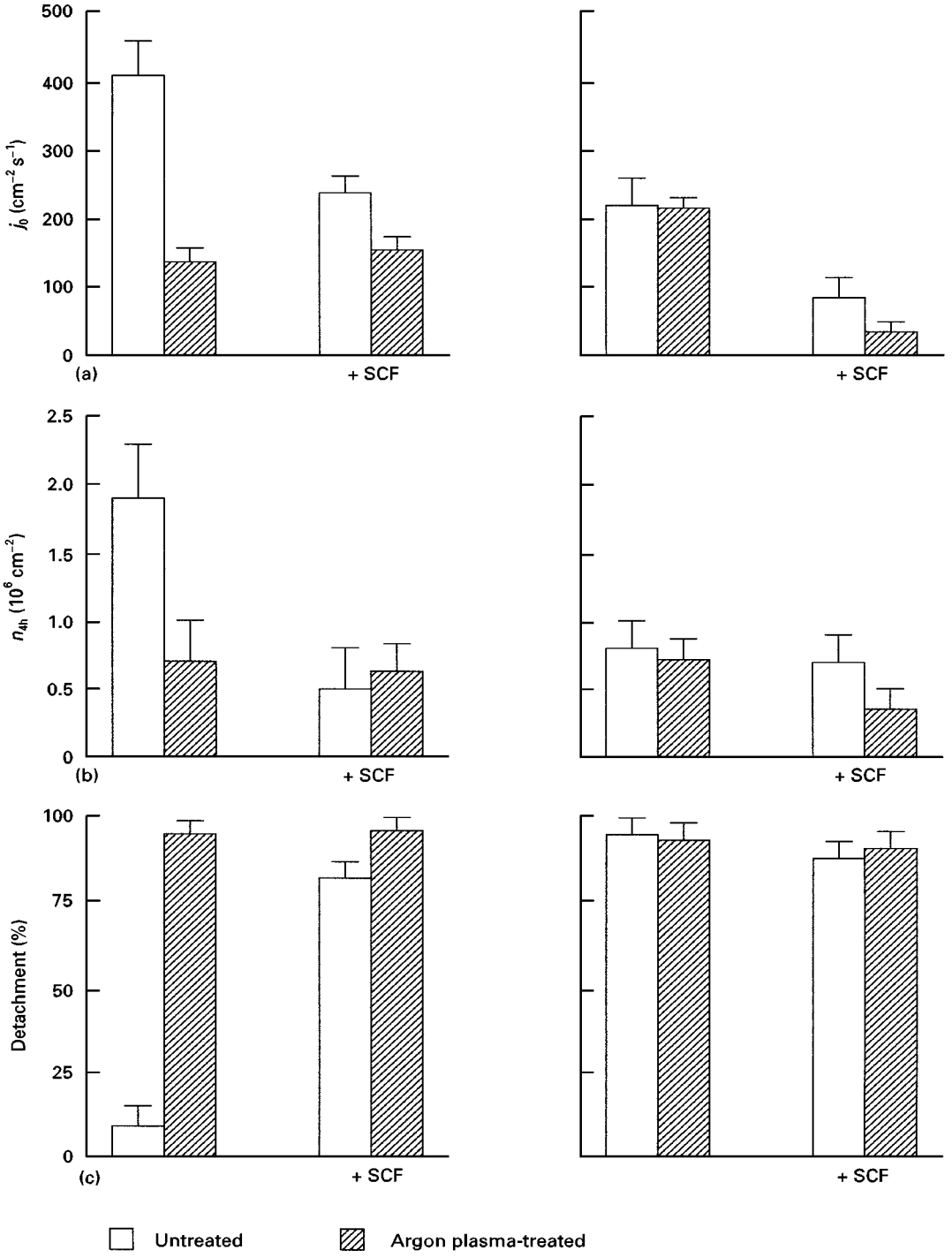

Figure 4 (a) Initial deposition rates, j_0, (b) numbers of adhering yeasts after 4 h, n_{4h}, and (c) percentages of yeasts detached after passing an air bubble through the flow chamber to argon plasma-treated, hydrophilized and original, hydrophobic silicone rubber in the absence and presence of a salivary conditioning film (SCF), for two yeast strains; *C. tropicalis* GB 9/9 (left hand panel) and *C. albicans* GB 1/2 (right hand panel).

that biofilm formation in the oro-pharyngeal region is governed by the hydrophobicity of the surfaces exposed.

Also, *in vitro* studies on oral streptococcal adhesion to materials with and without a salivary conditioning film, and comparisons with *in vivo* evaluation of dental plaque formation, revealed such discrepancies [28–30]. In these studies, oral streptococcal adhesion to surfaces *in vitro* in the absence of a salivary conditioning film were governed by the hydrophobicity of the substratum surfaces, but when surfaces were covered by a salivary conditioning film, all surfaces attracted more or less similar numbers of adhering streptococci [28]. Dental plaque formation over a 9 d time period on these materials when glued on the front incisors of human volunteers, however, showed far less plaque on hydrophobic than on hydrophilic materials [30].

There are several reasons why *in vitro* and *in vivo* evaluation of the fouling properties of biomaterials surfaces might give contradictory results:

(i) the number of strains and species occurring *in vivo* and their variability in cell surface properties is much larger than can be evaluated *in vitro*;

(ii) co-adhesion phenomena between bacteria, yeasts as well as between yeasts and bacteria

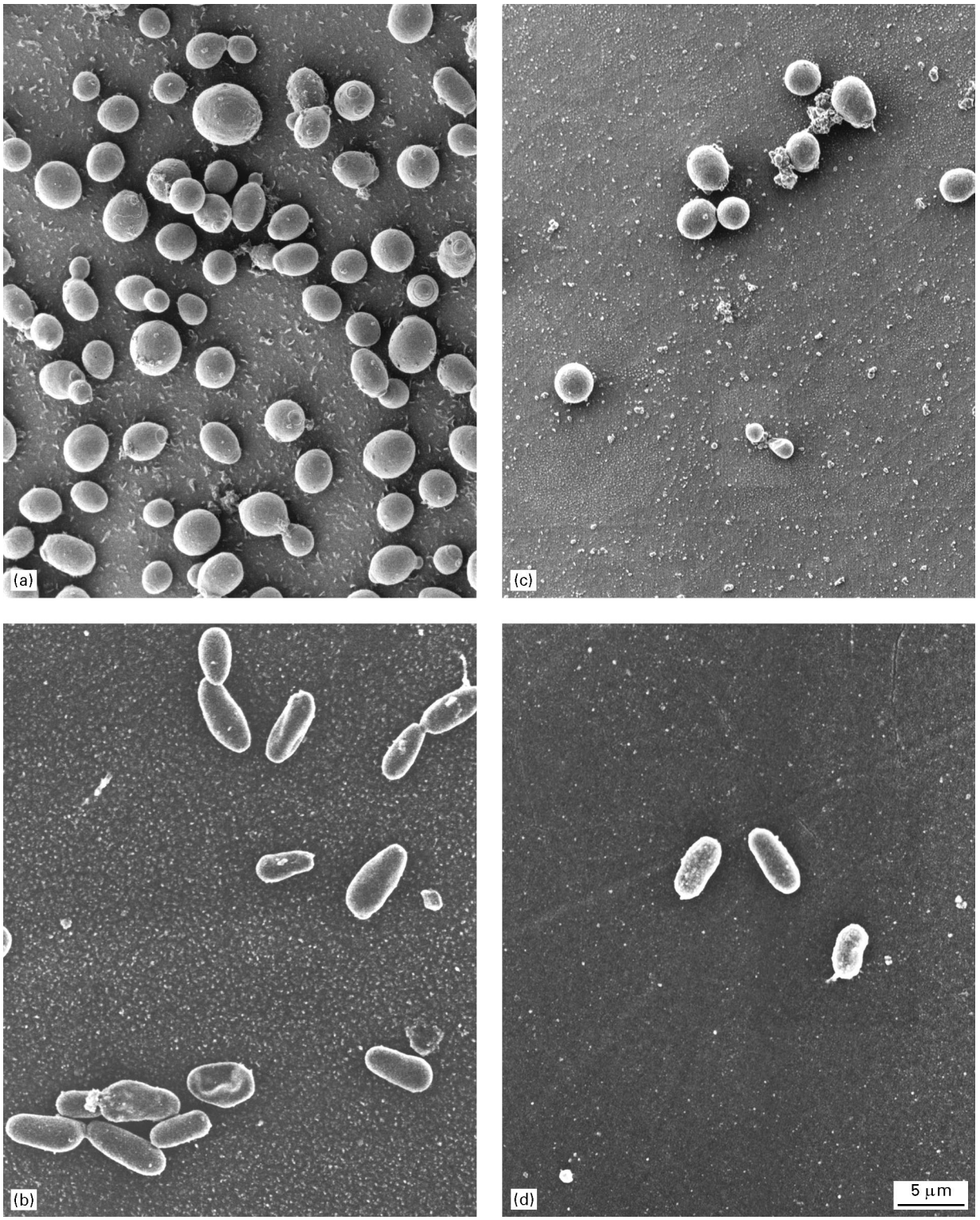

Figure 5 Scanning electron micrographs of (c, d) argon plasma-treated, hydrophilized (a, b) and original, hydrophobic silicone rubber, after growth of (a, c) *C. albicans* GB 1/2- or (b, d) *C. tropicalis* GB 9/9 in a modified Robbins device under dynamic nutrient conditions.

occur *in vivo* [31–34] but make *in vitro* evaluation even more difficult.

(iii) the conditions in the oro-pharyngeal activity are highly dynamic with regard to nutrient availability, temperature, humidity and shear.

The existence of periods with low and high shear, like during swallowing, eating and drinking, puts special emphasis on the initially adhering micro-organisms as a link between the biomaterial and the biofilm as a whole [35]. It has been suggested that during periods of low shear, micro-organisms adhere to the outer layer of the conditioning film, which may be relatively similar on different biomaterials, explaining why the microbial compositions on hydrophilized and hydrophobic valves are more or less identical. However, during high shear periods, the cohesivity of the conditioning film, which is strongly dependent upon the biomaterials hydrophobicity, may not be sufficient, yielding detachment of the

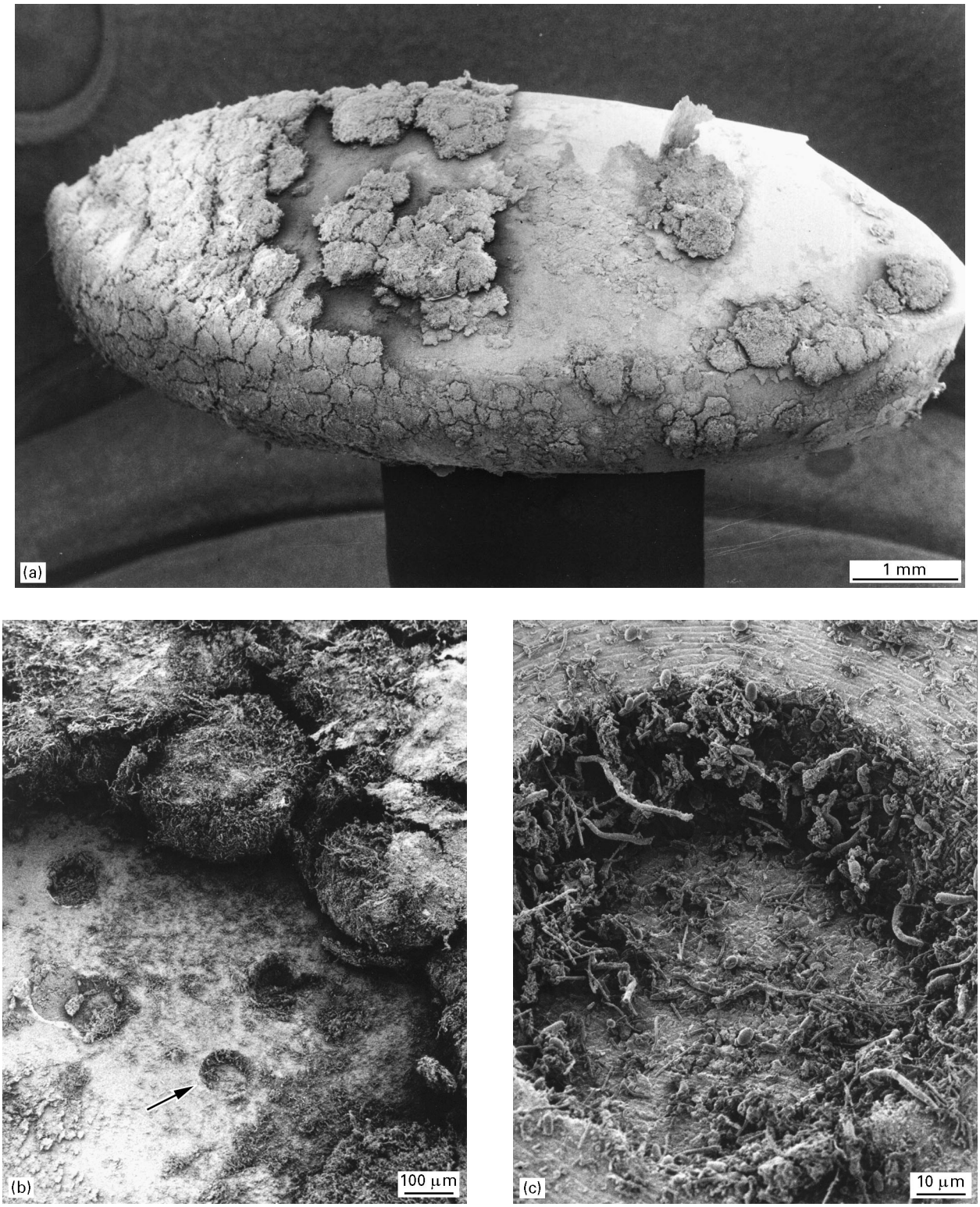

Figure 6 Scanning electron micrographs of partly hydrophilized "Groningen Button" voice prostheses removed from laryngectomized patients after a planned evaluation period of 4 wk. (a) Low-magnification electron micrograph showing differential biofilm formation on the hydrophilized (left) and original, hydrophobic side of (right) of the oesophageal flange of a prosthesis. (b) Detail of a heavily colonized prosthesis (hydrophilized side) with ingrowing micro-organisms, yielding deterioration of the silicone rubber surface. (c) Crater-like defect in the hydrophilized silicone rubber due to microbial ingrowth.

entire biofilm on top of it, as evidently occurs more readily on the hydrophobic side of a valve than on the hydrophilized side.

Nevertheless, this study convincingly demonstrates that biofilm formation on surfaces *in vivo* is governed by substratum hydrophobicity. For an improved anti-fouling performance of voice prostheses, increasing the hydrophobicity of the silicone rubber, like, for example, by adsorption of fluorocarbons, could be a possibility. Fluorocarbon surfaces (i.e. Teflon) are slightly more hydrophobic than silicone rubber and hardly attracted any dental plaque during 9 d exposure to dynamic conditions of the human oral cavity [30].

TABLE II Microbial strains and species isolated from the original, hydrophobic (SR) and argon plasma-treated (Ar), hydrophilized side of "Groningen Button" silicone rubber voice prostheses removed from laryngectomized patients, after a planned evaluation period of 4 wk. "X" indicates detection of a strain or species

	Patient A		Patient C		Patient D		Patient F		Patient G	
	SR	Ar	SR	Ar	SR	Ar	SR	Ar	SR	Ar
Yeast strains										
Candida albicans	X	X		X	X	X	X	X	X	X
Candida glabrata			X	X						
Candida inconspicua			X	X						
Candida krusei			X	X			X	X		
Candida lusitania			X	X						
Bacterial strains										
CDC group E (act. spp)			X		X		X			
Lactobacillus species					X	X	X	X		
Micrococcus luteus		X								
Salmonella subspecies				X						
Serratia marcescens	X	X								
Staphylococcal strains	X				X	X	X	X	X	X
Streptococcal strains	X	X					X	X		

5. Conclusions

There are two main conclusions to be drawn from the results of this study.

1. Biofilm formation on voice prostheses in the oro-pharyngeal region is governed by the hydrophobicity of the surfaces exposed and occurs more readily on hydrophilic than on hydrophobic biomaterials surfaces, in contrast with expectations based on *in vitro* work.

2. *In vitro* studies on the fouling properties of biomaterials should be modified to include the dynamic conditions occurring *in vivo* in order to increase their predictive value with respect to the clinical performance of a biomaterial.

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