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Adhesion of slime producing *Staphylococcus epidermidis* strains to PVC and diamond-like carbon/silver/fluorinated coatings

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Abstract *Staphylococcus epidermidis* has emerged as a pathogen associated with infections of implanted medical devices. Bacterial adhesion is a crucial step in infection on biomaterial surfaces. To quantitatively determine the relationship between poly (vinyl chloride) (PVC) surface properties and bacterial adhesion, we have compared attachment of slime-producing *S. epidermidis* strains on PVC and various coatings under flow conditions. Bacterial adhesion and colonization was quantified by counting the viable organisms on the adherent surface as well as by scanning electron microscopy, epifluorescence microscopy and atomic force microscopy. Fluorination of the PVC surface encourages *S. epidermidis* adhesion whereas; diamond-like carbon (DLC) and especially silver (Ag) coatings seem to inhibit its adhesion. In most materials, the number of adherent bacteria decreased with the increase of shear rate. These results indicate that bacterial adhesion is influenced by the chemical properties of the polymeric surfaces, the surface roughness and the associated flow conditions.

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1. Introduction

Infection remains a major impediment to the long-term use of implanted or intravascular devices [1,2]. *Staphylococcus epidermidis* is one of the most common bacterial species isolated from medical device infections [3]. Despite having low pathogenic potential under normal circumstances, this microorganism has evolved into being the leading cause of infection in the immunocompromised host or in the presence of a medical device. There are two main characteristics of *S. epidermidis* that allow persistence of infection. These are the ability of the bacteria to adhere to surfaces in multilayered cell clusters, followed by the production of a mucoid substance more commonly known as slime. The adherent bacteria and slime are collectively known as biofilm [4].

Bacterial adhesion to biomaterial surfaces is an essential step in the pathogenesis of these infections [5]; however, the molecular and physical interactions that govern bacterial adhesion to biomaterials are not completely understood, although surface chemistry, physical characteristics and shear rate all play a role. Both specific (i.e., receptor-ligand) and non-nonspecific (i.e., colloidal-type) interactions contribute to the ability of the bacterial cell to attach to (or to resist detachment from) the biomaterial surface [6,7]. The relative contributions of specific and non-specific mechanisms are likely to depend on the surface properties of biomaterial as well as the associated flow conditions.

The initial adhesion phase is largely governed by physicochemical interactions between the bacteria and substrate [8]. Once embedded in the biofilm layer the microorganisms are protected from the host defense mechanisms and from external agents such as drug treatments [9,10]. In many cases the only effective therapy for these infections is removal and replacement of the device. In this respect, the ability to control the development and the virulence of the bacterial infections

has deep consequences on patient discomfort and on the costs of the medical cures. One possibility to reduce bacterial adhesion to medical devices is to alter the physicochemical interactions between bacteria and substrate [8]. This can be done by modifying the surface of existing biomaterials or devices. As a result, a significant number of studies on improving the antibacterial properties of materials have focused on surface modification. Diamond-like carbon (DLC) coatings [11], fluorinated films [12], silver (Ag) coatings [13], surface thiocyanation [14], and surfaces modified by various gas plasmas, such as oxygen [15] have been proposed.

DLC is a form of amorphous carbon or hydrogenated amorphous carbon and is used as a protective coating with medical, tool making and optical applications due to its biocompatibility, high hardness, good scratch and wear resistance, and good chemical inertness [16,17]. It has previously been shown that DLC deposited on polyethylene terepthalate (PET) reduced *S. epidermidis* and *S. aureus* adhesion, since the adhesion efficiency of *S. aureus* on the coated PET was about 16% of that on the untreated PET surface, and the adherent bacterial concentration of *S. epidermidis* on the coated PET was about 1/6 of that of the PET surface [11]. Fluorinated films have shown good haemocompatibility, since they do not modify platelet behavior, and they are not cytotoxic [12]. Ag^+ ions exhibit good antibacterial properties by binding strongly to electron donor groups on biological molecules containing sulfur, oxygen or nitrogen, and by displacing other essential metal ions such as Ca^{2+} or Zn^{2+}. These result in blocking respiration and inhibiting hydrogen transfer, which is the main energy transfer system of bacteria, and thus bacterial death results. Therefore, up to 2 orders of magnitude reduction in *S. epidermidis* adhesion was achieved for Ag coatings on polyurethane [13].

The above mentioned studies [11,12,13,14,15] involved static experiments. However the process of bacterial adhesion to indwelling medical devices is associated in most cases with flow of body fluids. Within the intravascular space, the fluid shear rate on the biomaterial may also govern attachment and detachment of bacteria. Physiological shear rates can range between 40 s^{-1} and 2000 s^{-1} for stable laminar flow vessels, with much higher shear rates possible in turbulent flow or at the vessel entrances and bifurcations [18]. Because stable adhesion requires both attachment and resistance to detachment under these shear conditions, the sensitivity of bacterial cell attachment and detachment to shear rate is an important factor in determining the likelihood of cell adhesion [19].

The aim of this study was to evaluate two slime producing *S. epidermidis* strains adhesion to PVC and various coatings incorporating DLC, fluorinated and Ag. Furthermore, the relationship between surface chemistry and bacterial "attachment"/"detachment" process was investigated under different flow conditions. PVC was chosen because it is an inexpensive biomaterial widely used in medical devices and, to our knowledge, the use of DLC, fluorinated or Ag coatings on it, to render it more bacterial resistant under flow conditions, has not been reported. Surface modification of PVC was achieved by both radio frequency (rf) and atom beam plasma discharges. The latter plasma source was used to deposit diamond-like carbon (DLC) coatings and when combined with a magnetron sputtering source [13] was used to deposit both silver (Ag) and Ag-DLC coatings. The rf source was also used to deposit DLC coatings in addition to fluorinated surfaces.

2. Materials and methods

2.1. Bacterial strains and growth media

The strains of *S. epidermidis* that were used in the experiments were the reference ATCC 35984 slime producing and the clinical strain GRE506 isolated from a catheter-related infection of hospitalized patient at the Microbiology Laboratory of the University Hospital of Patras. The clinical strain was characterized by biochemical tests (API Staph System, BioMerieux, SA Lyon, France) and molecular typing methods [20]. Slime production was investigated by the method described by Ishak *et al.* [21]. Microorganisms were kept at −70°C, in 70% Tryptic Soy Broth (TSB, Difco Laboratories, Detroit, USA) and 30% of 50% glycerol solution.

Before each experiment, 10 μl of the frozen bacterial suspension was subcultured onto Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, USA) for 24 h at 37°C. Stationary phase cells were obtained by incubating two to three colonies, from the TSA, in 5 ml TSB for 18 h at 37°C in a rotary shaker at 120 r.p.m. Cells were harvested by centrifugation at 4000 r.p.m, at 4°C for 10 min, washed twice with PBS, pH 7.4 and finally resuspended in PBS buffer at a concentration of 3×10^8 cfu/ml (according to McFarland standard, BioMerieux, SA Lyon, France). Clusters of bacteria were dispersed by aspirating and expelling each suspension twice through a sterile 25 gauge steel needle, attached to a syringe.

2.2. Buffered media

The fluid media used in our flow experiments was Dulbecco's phosphate buffered saline (Gibco BRL, Scotland, UK), supplemented with 0.1 mg/ml $MgCl_2$ and 0.1 mg/ml $CaCl_2$, at pH 7.4. Hereafter, this solution will be referred to as "buffer." The buffer was filtered and degassed before use.

2.3. Plasma surface modifications

The substrate used for plasma modification and bacterial adhesion assays was medical grade PVC, used in the

Table 1 Description and number of materials used

Sample	Number of attachment experiments	Number of detachment experiments
[a]PVC unmodified, unsterilized	4	4
[b]PVC unmodified, EtO sterilized	4	4
[c]Fluorinated	3	3
[d]DLC (A.B.)	4	4
[e]DLC (rf)	4	4
[f]Ag Thin	2	2
[g]Ag Thick	3	4
[h]Ag/DLC (A.B.)	2	2

[a]PVC unmodified, unsterilized used for comparison reasons and as substrate for plasma modifications.
[b]PVC unmodified, sterilized with ethylene oxide.
[c]Fluorinated PVC deposited by radio frequency (rf) plasma discharges. No visible coating but decrease of surface energy, so chemical modification-fluorination.
[d]Diamond-like Carbon (DLC) coated PVC deposited by Atom Beam (A. B.) plasma discharges.
[e]Diamond-like Carbon (DLC) coated PVC deposited by Radiofrequency (rf) plasma discharges.
[f]Thin Silver (Ag) coated PVC deposited by the combined Atom Beam/Magnetron Sputtering Source, after 20 s.
[g]Thick Silver (Ag) coated PVC deposited by the combined Atom Beam/Magnetron Sputtering Source, after 70 s.
[h]Silver (Ag)/Diamond-like Carbon (DLC) coated PVC deposited by the combined Atom Beam/Magnetron Sputtering Source.

manufacture of blood bags (Ergo, Athens). PVC sheets were cut into pieces of 44 mm diameter and were then modified. PVC sterilized with ethylene oxide was used as control. All the other samples were sterilized during plasma modification process and no contamination was ever observed after inoculation of the samples onto solid media. Table 1 describes the materials used.

The fluorination treatments and DLC (rf) coatings were carried out using a 13.56 MHz plasma in a cylindrical stainless steel chamber, as described elsewhere [16]. The PVC substrates were placed on a biased 10 cm diameter cylindrical steel electrode. The fluorinated surfaces were obtained from CF_4/H_2, gas mixtures, while the DLC (rf) coatings were obtained from C_2H_2/He gas mixtures. The deposition pressure was 15 Pa.

DLC (A.B.) films were deposited using a neutral atom beam (Saddle Field) plasma source [22,23] onto PVC substrates from C_2H_2 [16]. The substrates were treated in an argon plasma for 60 seconds prior to coating deposition, in order to enhance adhesion. The source operated at 1000 V and at a pressure of 0.1 Pa.

Coatings containing Ag were deposited by the combined atom beam/magnetron sputtering source [13]. The advantage of this combination was that the metal could be deposited at relatively low substrate temperature, which is very important

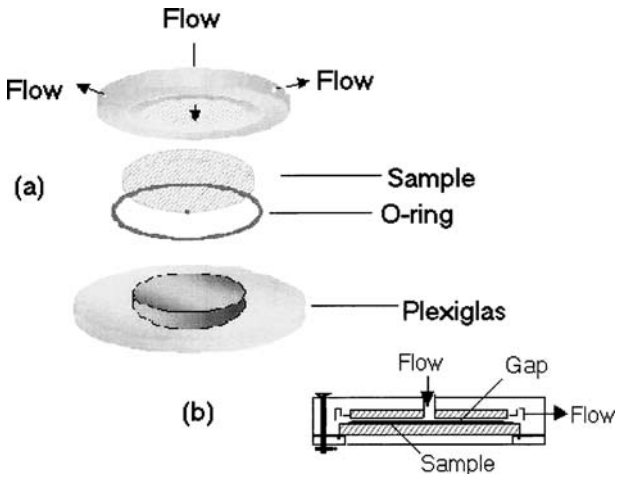

Fig. 1 Radial flow chamber. (a) Dismantled. (b) Assembled.

for the coating of thermally sensitive polymer substrates. The atom beam (argon plasma current 120 mA at 0.1 Pa) was used both to activate the polymer surface for 60 s prior to coating deposition and also during the silver deposition process. The polymer samples were mounted approximately 35 cm from this source. The Ag coatings were deposited from the 6.5 cm diameter cylindrical target with a deposition current of 0.4 A.

2.4. Radial flow chamber

A radial flow chamber (RFC) was constructed for this study (modified version of the one described by Dickinson and Cooper, 1995) [19] and is shown in Figure 1. Briefly, the polymer film (sample) is sandwiched between two Plexiglas disks in such a way that a circular space of 44 mm diameter and 0.2 mm height is formed. The fluid enters through a centrally located inlet port at the upper disc and exits through three equally spaced ports after coming into contact with the sample's surface. It is collected in a surrounding trough, and by the pumping action follows the opposite path. This cycle repeats itself for 150 min for the "attachment" experiments and for 140 min for the "detachment" experiments. The fluid dynamics between the disks are well defined, such that the shear stress on the surface is inversely proportional to the radial position. In particularly, for a given volumetric flow rate (Q), the shear rate on the collector surface (S) is inversely proportional to the radial position from the inlet port r, and is calculated from the relation: $S = 3Q/\pi rh^2$ (s^{-1}), where h is the gap width.

In the "attachment" experiments, bacteria in suspension (3×10^8 cfu/ml) flowed through the RFC at a flow rate of 4 ml/min for 150 min, corresponding to shear rates ranging between 50 s^{-1} and 200 s^{-1}. The bacterial "detachment" experiments were conducted by first injecting the bacterial suspension (3×10^8 cfu/ml) into the RFC and allowing the bacteria to settle for 10 min before initiating flow. A high

flow rate of 40 ml/min for 140 min was used to obtain significant detachment corresponding to a range of shear rates from approximately 500 s^{-1} to 2000 s^{-1}.

2.5. Contact angle measurements

Contact angle measurements were performed on unmodified and modified PVC sheets by the sessile drop technique using a Dataphysics OCA measurement system. Ultra pure water was used and contact angles were read on both sides of each static droplet. Values reported are the average of measurements on three water drops.

2.6. Coating thickness

Coating thickness was obtained using the optical profilometry technique. Measurements were made of the step heights between the coated and uncoated areas of glass slide samples mounted on the substrate holder for the PVC sheet.

2.7. Atomic force microscopy

Surface topography of native and plasma modified PVC was examined by contact mode atomic force microscopy using a commercial Multimode AFM (Nanoscope III, Digital Instruments, Santa Barbara, CA). The system used is equipped with a piezoelectric scanner to allow a maximum scan size of 100 μm. Standard contact mode cantilevers and integrated silicon nitride tips (Digital Instruments, Santa Barbara, CA) were used. The surfaces were analyzed by measuring the average surface roughness (Ra) after a first order flattening procedure of the height data from the 10 $\mu m \times 10\,\mu$m area images. The Ra is the main height as calculated over the entire measured area and is given by the mean deviation of the data from the average of the data. It is typically used to describe the roughness of machined surfaces and it is useful for detecting general variations in overall profile height characteristics.

2.8. Analysis of adherent bacteria

2.8.1. Colony forming units counting

After adhesion experiments in the flow chamber for 150 min, each PVC sample was gently rinsed with 10 ml of PBS to remove non-adherent or loosely adherent bacteria and then pieces (1 cm^2) located at specific radial distances from the center of the disk, corresponding to shear rate values of approximately 50 s^{-1} and 200 s^{-1} for "attachment" experiments and of 500 s^{-1} and 2000 s^{-1} for "detachment" experiments, were cut and placed into a tube with 5 ml of fresh sterile PBS. All the tubes were sonicated for 10 min in an ultrasonic cleaner; then 10-fold serial dilutions of the sonicated solutions were inoculated onto TSA plates, and the numbers of adherent bacterial colonies were counted after 18 h of incubation at 37°C. Material samples were also plated on TSA plates after the sonication procedure in order to check if all bacteria were removed by sonication. In the cases that there were still bacteria on the samples, the bacterial colonies were counted and added to the PBS bacterial counts. In parallel, bacterial suspensions, before and after adhesion experiments were 10-fold diluted and inoculated onto TSA plates and the numbers of bacterial colonies were counted after 18 h of incubation at 37°C. These results were compared with those obtained from the sonicated solutions (adherent bacteria) in order to evaluate the decrease in the number of bacteria in the bacterial suspensions due to the adherent bacteria [24].

2.8.2. Scanning electron microscopy (SEM)

After adhesion experiments in the flow chamber for 150 min, each PVC sample was gently rinsed with 10 ml of PBS to remove non-adherent or loosely adherent bacteria and then fixed for 20 min with 2.5% glutaraldehyde (Sigma) in PBS [24]. Each sample was subsequently dehydrated by several passages in ethanol-water solutions for 20 min each using increasing concentrations of ethanol up to 100%. After sputter-coating with gold, the samples were then investigated with a JEOL-JSM 6300 Scanning Electron Microscope (Accelerating Voltage: 20 kV). Adherent bacteria were counted in fields located at specific radial distances from the center of the disk corresponding to shear rate values of approximately 50 s^{-1}, 200 s^{-1}, 500 s^{-1} and 2000 s^{-1}. Three fields for each shear rate value, for each sample, were chosen randomly to eliminate the possible uneven distribution of bacteria and magnifications of $\times$2000 used to count bacteria. The total numbers of adherent bacteria counted on each field were then divided with the image area to give the density of bacteria per mm^2 of surface.

2.8.3. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) detects nucleic acid sequences by a fluorescent-labeled probe that hybridizes specifically to its complementary target sequence within the intact cell. In order to detect, identify and enumerate the microbial cells which were attached to the polymer surface, we performed the hybridization procedure by using the DNA probe EUB338 specific for all eubacteria. The probe was a universal oligonucleotide complementary to virtually all the 16S rRNA (positions 1392 to 1406), labeled on its 3′ end with cy3, processing the following sequence: 5′-ACGGGCGGTGTGT[G/A]C-3′. (Laboratory of Microchemistry, Institute of Technology and Research, Crete, Greece) [25].

After adhesion experiments in the flow chamber for 150 min, each sample was gently rinsed with 10 ml of PBS to remove non-adherent or loosely adherent bacteria. Cells were fixed by air-drying at 4°C overnight. For permealization, the cells were treated with an enzyme mix (750 μg of lysostaphin per ml, 5 mg of lysozyme per ml, 50 mM sodium phosphate, 0.05% saponin) for 1 h at 37°C. RNA fixation was achieved in UV Stratalinker (Stratagene, La Jolla, USA). Hybridization was performed in 500 μl of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl pH 7.5, 0.001% SDS, 43% formamide) with 50 ng of Cy3- or fluorescein-labeled probe EUB338 for 3 h at 43°C. Removal of the unbound probe and washings were performed at 43°C by flushing the specimen with several ml of wash solution (0.9 M NaCl, 50 mM sodium phosphate pH 7.0, 0.1% SDS) for 15 sec and then by thorough rinsing with distilled water. The spesimens were then air dried in the dark and stored at room temperature in the dark or viewed immediately by epifluorescence microscopy. Three fields for each shear rate value (50 s^{-1}, 200 s^{-1}, 500 s^{-1} and 2000 s^{-1}) for each experiment-sample were chosen randomly and photographed. Magnifications of × 1000 used to observe adherent bacteria.

2.8.4. Atomic force microscopy (AFM)

After adhesion experiments in the flow chamber for 150 min, each sample was gently rinsed with 10 ml of PBS to remove non-adherent or loosely adherent bacteria and then the samples were investigated with AFM in tapping mode, in order to reduce lateral forces that may destroy bacteria cells. Standard tapping mode cantilevers and integrated silicon tips (Digital Instruments, Santa Barbara, CA) were used [26].

2.9. Statistical analysis

The effects of surface chemistry and flow conditions on bacterial adhesion were statistically analyzed using SPSS 12.0 package for windows. One-way analysis of variance (ANOVA) and in particular Post-hoc comparisons of all possible combinations of group means were performed using the Scheffe Significant Difference test. In all cases $P < 0.05$ was chosen to denote the significance level.

3. Results

3.1. Contact angle measurements

The influence of the surface modifications on water contact angles carried out in this study is given in Table 2. The deposition of the DLC coatings resulted in a decrease in contact angle, the surface therefore became more hydrophilic. The contact angle for the Ag coating was similar to that of the uncoated PVC. In contrast the fluorinated PVC was more hydrophobic as demonstrated by the increase in contact angle.

Table 2 Water contact angle measurements (deg) for unmodified and plasma modified PVC, as described in section 2.5. Results are the means± SD of three measurements.

Polymers	Water contact angle (deg)
PVC Unmodified, Eto	105.5 ± 1.1
Fluorinated	141.05 ± 0.2
DLC (A.B.)	85.6 ± 4.8
DLC (rf)	88.8 ± 0.8
Ag Thick	109.6 ± 0.8

3.2. Atomic force microscopy

AFM images of the uncoated PVC substrate showed that the surface was very rough with deep trenches. The Ra measurements (Table 3) for 10 μm × 10 μm area images showed that the Ag coating and the fluorinated treatment resulted in Ra values which are significantly lower than those of the uncoated PVC. The DLC (rf) had a similar Ra value to that of the PVC, while the DLC (A.B.) had a significantly higher Ra value. This difference in Ra values between the two types of DLC coating is possibly due to differences in the structural composition of the coatings. The Atom Beam DLC has previously been shown to exhibit higher refractive indices than for DLC coatings deposited using the rf technique [16]. The higher refractive index DLC coatings exhibit superior mechanical properties, but may lead to a higher level of compressive stress, which in turn could lead to distortion of the polymer substrate.

Table 3 Average surface roughness (Ra) measurements by Atomic Force Microscopy of unmodified and plasma modified PVC for 10 μm x 10 μm area images, and coating thickness measurements. For 10 μm x 10 μm area images, results are the means ± SD of three measurements and Ra measurements are statistically different except from those for PVC unmodified-DLC (rf) and Fluorinated (rf)-Ag Thick.

Polymers	Ra(nm) (10 μm × 10 μm)	Coating Thickness (μm)
PVC Unmodified, Eto	29.2 ± 11.7	—
Fluorinated	19.5 ± 1.8	Not visible
DLC (A.B.)	46.8 ± 2.9	0.10
DLC (rf)	25.1 ± 1.6	0.30
Ag Thin	—*	0.01
Ag Thin	15.6 ± 2.4	0.04
Ag/DLC (A.B.)	—*	0.10

* Not measured.

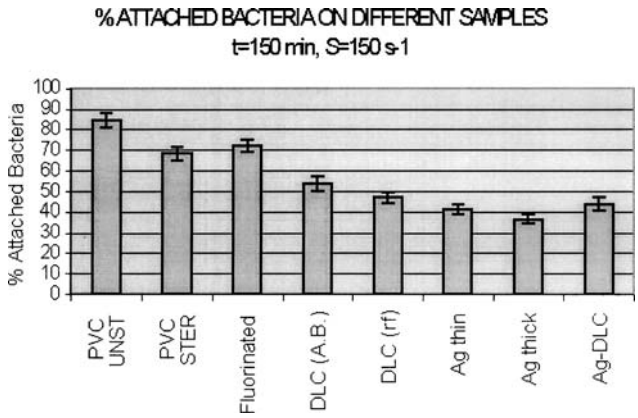

Fig. 2 Percentage (%) of attached bacteria on PVC and DLC/Ag/Fluorinated Coatings, under $S = 150\ s^{-1}$. All the results are significantly different except from those for PVC ster-Fluorinated, DLC (A.B.)-DLC (rf)-Ag/DLC-Ag Thin and Ag Thin-Ag Thick.

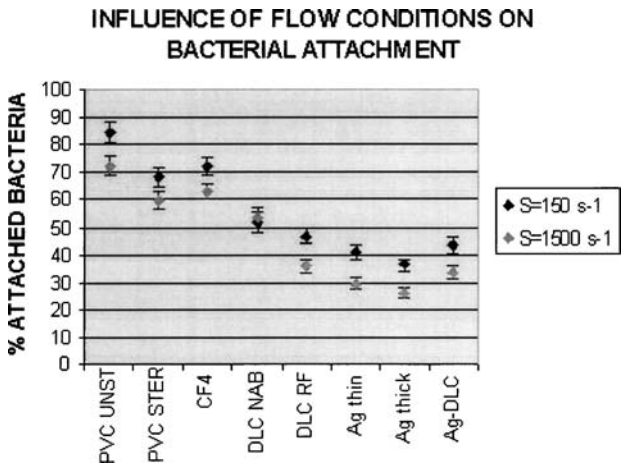

Fig. 3 Influence of flow conditions on bacterial attachment. All the results at $S = 1500\ s^{-1}$ are significantly different except from those for PVC ster-Fluorinated-DLC (A.B.) and DLC (rf)-Ag/DLC-Ag Thin. Between $S = 150\ s^{-1}$ and $S = 1500\ s^{-1}$, the results are significantly different except from those for DLC (A.B.).

3.3. Coating thickness

Table 3 presents the thickness measurements of the various coatings. DLC (rf) was the thickest coating followed by DLC (A.B.) and Ag/DLC. Ag Thick and especially Ag Thin presented very low thickness. In the case of the fluorination treatment, no visible coating was formed, however, the increase in the water contact angle, indicated that this treatment involved a chemical modification-fluorination of the polymer rather than the deposition of a coating.

3.4. Bacterial adhesion

Figure 2 shows the percentage (%) of attached bacterial cells of the reference strain ATCC 35984, in relation to the density of the bacterial suspension introduced in the radial flow chamber, on the various substrates after 150 min of incubation of the sample under shear rate $S = 150\ s^{-1}$. This was calculated by counting the colony forming units (CFU) of the viable organisms on the adherent surface. These results were in agreement with those obtained by counting CFU of the viable organisms in the suspension solutions before and after adhesion experiments; increase in the number of adherent bacteria conforms to a decrease of the bacterial counts in the suspensions. DLC (rf), Ag Thin and especially Ag Thick significantly decreased the number of adherent bacteria. No significant difference was observed in the adhesion

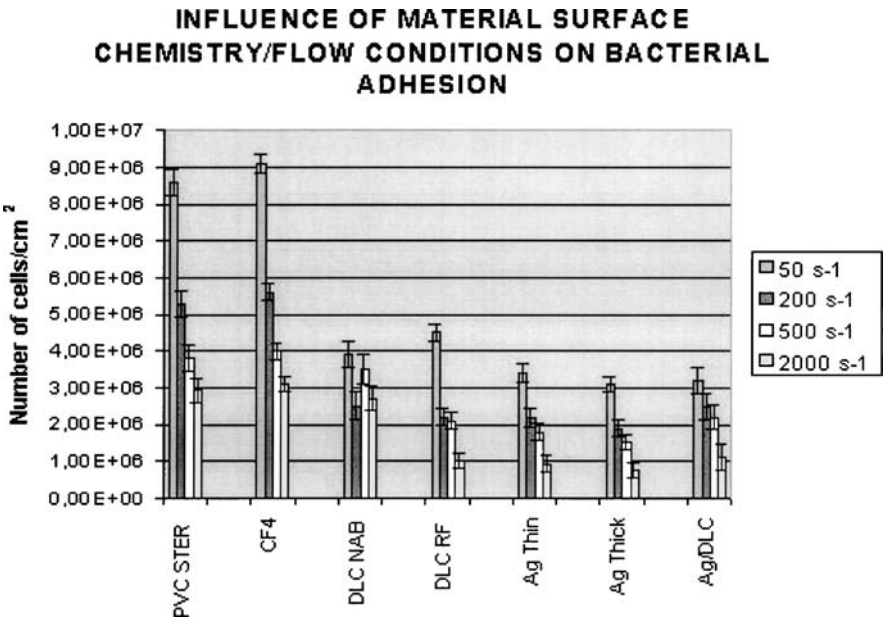

Fig. 4 Extent of bacterial adhesion on PVC and DLC/Ag/CF_4 Coatings. All the results at $S = 50\ s^{-1}$ and $S = 200\ s^{-1}$ are significantly different except from those for PVC ster-Fluorinated, DLC (A.B.)-DLC (rf)-Ag/DLC-Ag Thin and Ag Thin-Ag Thick. At $S = 500\ s^{-1}$ and $S = 2000\ s^{-1}$, all the results are significantly different except from those for PVC ster-Fluorinated-DLC (A.B.) and DLC (rf)-Ag/DLC-Ag Thin. The results between $S = 50\ s^{-1}$ and $S = 200\ s^{-1}$, $S = 50\ s^{-1}$ and $S = 500\ s^{-1}$, $S = 50\ s^{-1}$ and $S = 2000\ s^{-1}$ and $S=200\ s^{-1}$ and $S = 2000\ s^{-1}$ are statistically different [except from DLC (A.B.)], but not between $S = 200\ s^{-1}$ and $S = 500\ s^{-1}$, and $S = 500\ s^{-1}$ and $S = 2000\ s^{-1}$.

experiments between the two slime-producing strains, the reference and the clinical one (data not shown).

Figure 3 shows the effect of flow conditions on bacterial adhesion to the various substrates after 150 min of contact of the bacterial suspension with the sample. In most materials, except DLC (A.B.), the number of adherent bacteria significantly decreased with the increase of shear rate from 150 s^{-1} to 1500 s^{-1}.

Figure 4 shows the combined effect of PVC surface modification and shear rate on bacterial adhesion. A decrease

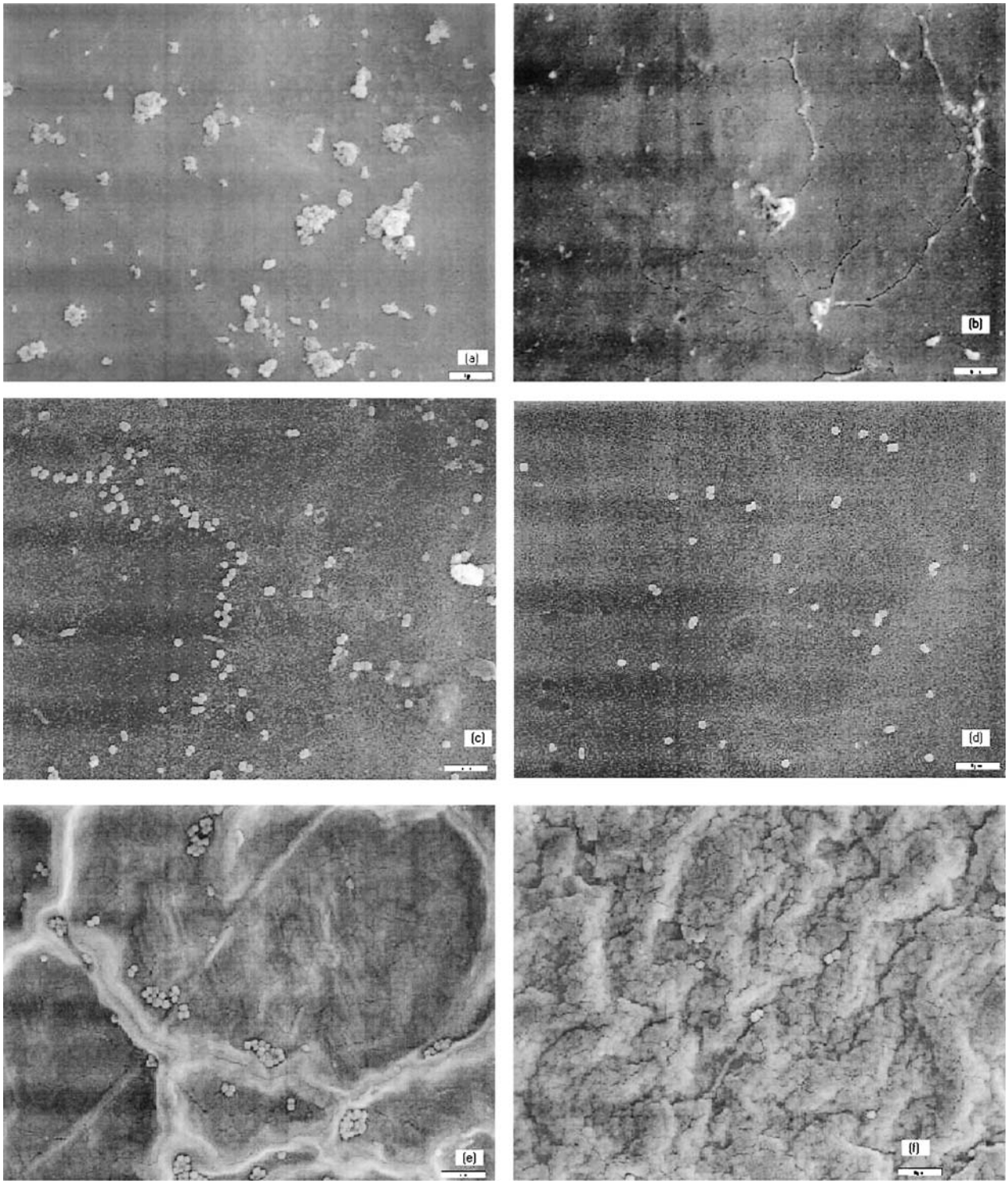

Fig. 5 SEM images showing bacterial adhesion on PVC and Fluorinated, DLC, Ag Coatings under S = 50 s^{-1} (a, c, e, g) and under S = 2000 s^{-1} (b, d, f, h) respectively (The bar in all SEM images is 5 μm, × 2000). (*continued*)

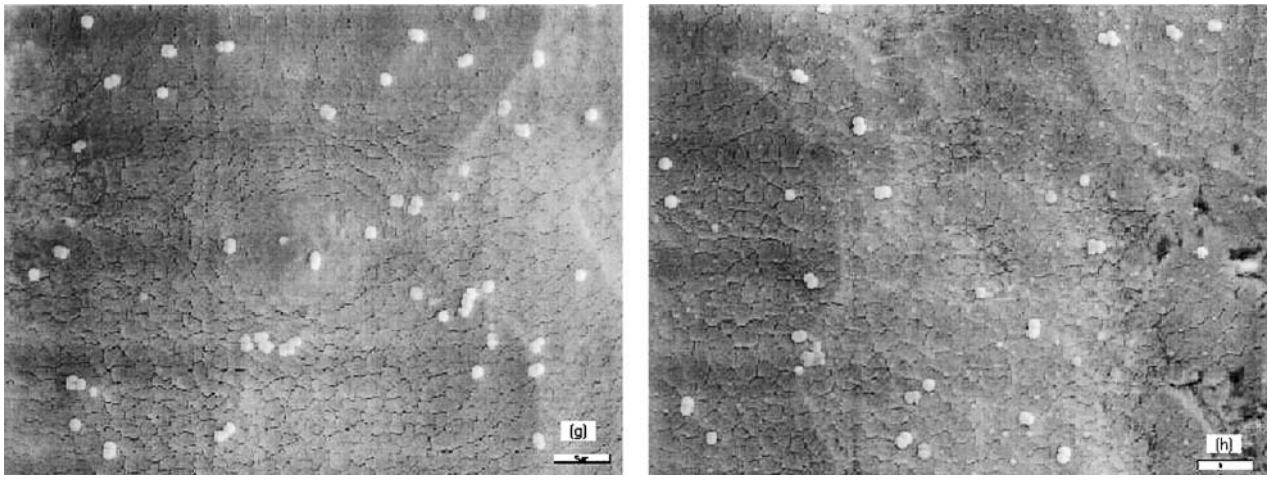

Fig. 5 (*Continued*)

for almost all materials in the number of attached bacteria was observed, as counted by SEM and epifluorescence microscope images, when the shear rate increased from 50 s^{-1} to 200 s^{-1} or 500 s^{-1} and especially when it reached 2000 s^{-1}. The only material that exhibited a different behavior was DLC (A.B.). Moreover, all coatings, except fluorinated, decrease bacterial adhesion.

Figure 5 shows the adherent bacteria morphology observed by SEM after bacterial-biomaterial contact under the influence of flow conditions. An interesting observation is that in the case of most substrates, except Ag, and especially in the case of PVC sterilized with ethylene oxide, bacterial aggregations and slime production can be observed.

Figure 6 shows SEM, AFM and Epifluorescence Microscopy images of *S. epidermidis* deposition on DLC (A.B.) coating ($S = 50\ s^{-1}$). SEM and AFM provide the relative position of adherent bacteria in relation to the material topography, whereas the Epifluorescence Microscopy Image of the fluorescence in situ hybridized bacteria helped us to detect, identify and enumerate the adherent bacteria even on materials that had very similar configuration-contrast with the microbial cells, like DLC (A.B.) [25].

4. Discussion

The purpose of this study was to examine how the surface modifications of PVC through the deposition of DLC, DLC/Ag, and Ag coatings, as well as through fluorination, influenced the adhesion of *S. epidermidis*. Our results suggest that the chemical composition of the surface, its roughness as well as the test flow conditions influence *S. epidermidis* adhesion.

The decreased bacterial adhesion at higher flow rates is clearly demonstrated. Dickinson *et al.*, 1995, also found that the number of adherent *S. aureus* on various substrates decreased with the increase of shear rate [19], however Mohamed *et al.*, 2000, showed that there was an optimum flow rate for bacterial attachment reflecting the balance between the rate of delivery and the force acting on the attached bacteria [27]. In particular, it was found that in the case of the ligand/receptor *S. aureus* adhesion to collagen coated coverslips increased between shear rates 50–300 s^{-1} and decreased for shear rates higher than 500 s^{-1}. However, in the case of lower number of receptors/cell this optimum flow rate was not clear [27]. Our results, like those by Dickinson *et al.*, 1995 [19], did not show this optimum flow rate, probably because we had no specific ligand-receptor interactions between *S. epidermidis* and the various substrates. Therefore, higher shear rates result in higher detachment forces and in subsequently decreased number of adherent bacteria for all materials except DLC (A.B.). This could be explained by DLC (A.B.)'s increased Ra as bacteria preferentially stick to rough surfaces and especially to irregularities that conform their shapes in order to maximize bacteria-surface contact area and probably protect themselves from shear forces [28].

The influence of the different surface modifications is summarized as follows:

Silver and Silver-DLC coatings: The PVC surfaces coated with Ag exhibited the lowest level of bacterial adhesion due to the anti-bacterial activity of the silver cation (Ag^+). This effect was greater for the thicker silver coating since the antibacterial activity of Ag is dependent on the concentration of Ag^+ ions released from the coating. The Ra value for the Ag coated PVC was significantly lower than that of the uncoated polymer which would also help to decrease bacterial adhesion. It should be stressed that the surface roughness of the PVC is significantly greater than the thickness of the different coatings, thus prohibiting a homogenous coating in the deep crevices. This factor may explain the relatively high levels of bacterial adhesion even for the Ag coatings,

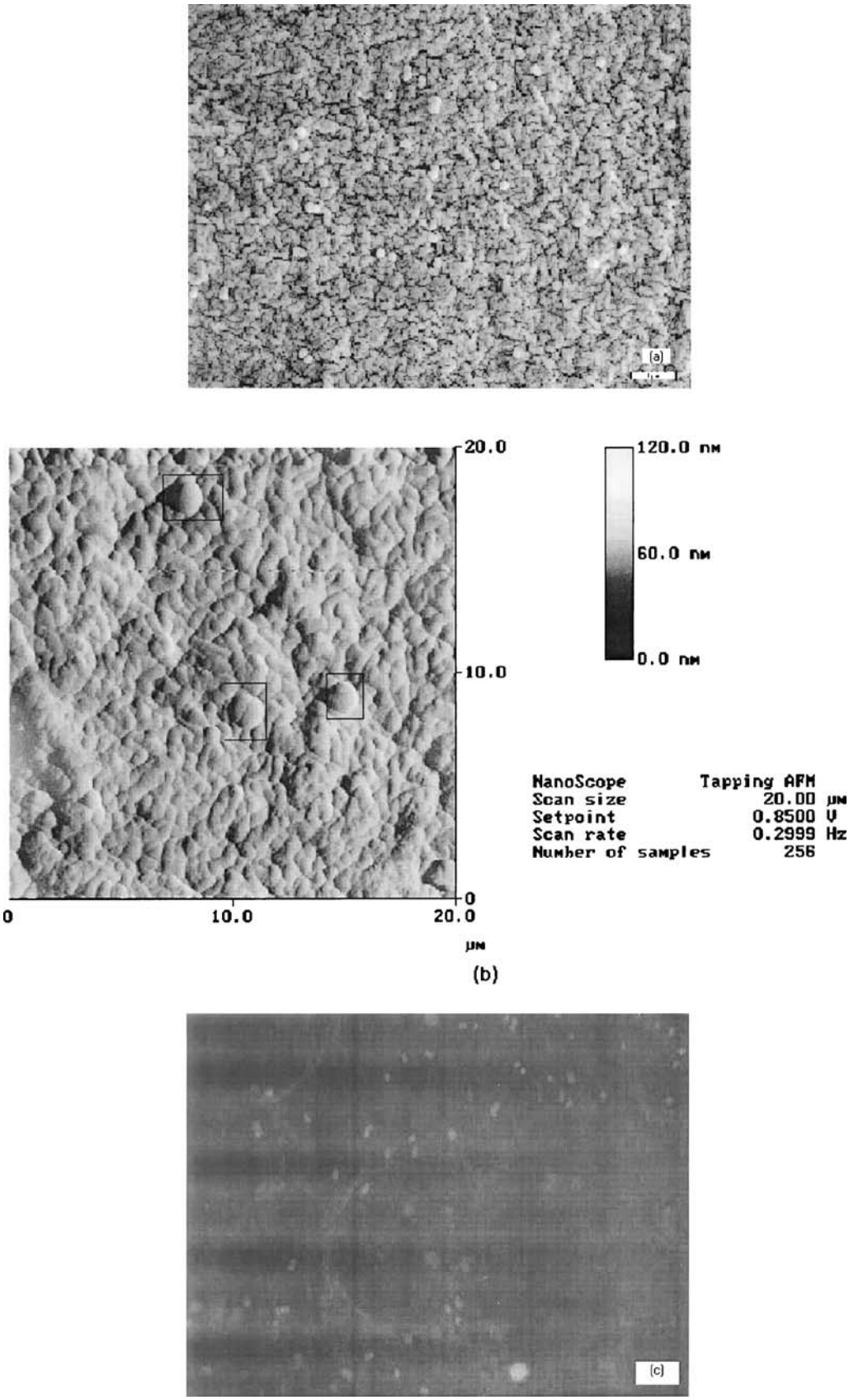

Fig. 6 Attachment experiment on DLC (A.B.) coating. a) SEM image (The bar is 5 μm, $\times$2000), b) AFM image and c) Epifluorescence Microscopy image (FISH) ($\times$ 1000).

in comparison to previous studies [13]. The adhesion of *S. epidermidis* to the Ag-DLC coating was greater than that for Ag but less than that observed for DLC coating. A possible explanation is that the available Ag surface required for the Ag^+ ions release is reduced with the combination coating.

DLC coatings: Two sources for the deposition of these coatings were investigated. Both sets of coatings exhibited lower levels of *S. epidermidis* adhesion than for the uncoated PVC probably due to their inertness and reduced hydrophobicity in comparison to PVC. The DLC (A.B.) retained larger numbers of attached bacteria, in comparison to DLC (rf), even under higher shear stress. This difference in behavior appears to be associated with the significantly lower surface roughness values of the rf compared with the Atom Beam deposited coatings.

Fluorinated surface: The fluorinated PVC exhibited a slightly higher level of *S. epidermidis* adhesion compared with the sterilized PVC. This increase is probably due to the increased hydrophobic properties of its surface since when both bacterial and biomaterial surfaces are hydrophobic, microbial adhesion is highly facilitated, whereas, if both bacterial and biomaterial surfaces are hydrophilic, microbial adhesion would proceed with difficulty [29]. In our case, *S. epidermidis* is a moderate hydrophobic bacterium (water contact angle $\approx$35) [30], therefore adhesion is favored to the most hydrophobic substrate, which, in our case, is the fluorinated surface. Pizzoferrato *et al.*, 1995, also showed that plasma fluorinated polyurethane caused a higher adhesion of *S. aureus* than did the uncoated surface [12], whereas modifications that incorporate hydrophilic functional groups such as thiocyanates [14] and oxygen [15] have shown that this kind of polymer surface modifications reduces bacterial adhesion. The relatively small increase in *S. epidermidis* adhesion observed for the fluorinated surface in this study however, may be due to the moderating effect associated with the reduction in surface roughness of the fluorinated PVC. The reduction in Ra would mean fewer sites for bacteria to adhere despite the more favorable hydrophobic surface.

Finally it is clear that the number of adherent bacteria, as they were counted by SEM (Fig. 4), are in good agreement with the results obtained by counting the viable organisms on the various substrates (Fig. 2). The small differences that are noted between the two methods could be explained by the fact that in case of big bacterial aggregations enumeration is difficult by SEM or even epifluorescence microscopy. Moreover, CFU counting counts alive bacteria, whereas SEM includes all bacteria irrespective of their metabolic activity level. This is probably the reason that CFU resulted in lower values than SEM in the case of Silver coatings, since Ag^+ ions exhibit antibacterial properties, therefore some bacteria were not alive, whereas it gave higher values than SEM in the case of plain PVC. Epifluorescence Microscopy on the other hand detected, identified and aided us to enumerate the adherent bacteria on materials that had similar configuration-contrast with the microbial cells [e.g. DLC (A.B.)]. Therefore, this combination of methods for the analysis of adherent bacteria was necessary as we had to deal with rough materials and, in some cases, with aggregated bacteria under the cover of a slimy matrix.

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