

# Fluid Flow and Sub-Bactericidal Release of Silver from Organic Nanocomposite Coatings Enhance *ica* Operon Expression in *Staphylococcus epidermidis*

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Received November 13<sup>th</sup>, 2013; revised December 13<sup>th</sup>, 2013; accepted December 23<sup>rd</sup>, 2013

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## ABSTRACT

The present study investigates the effect of a silver (Ag)-containing nanocomposite coating on *Staphylococcus epidermidis* adhesion and *icaA* gene expression. Bacterial interactions with organic coatings with and without Ag nanoclusters were assessed through a combination of both conventional phenotypic analysis, using microscopy, and genotypic analysis, using the relative reverse transcription Real-Time Polymerase Chain Reaction (RT-PCR). The results suggest that the incorporation of Ag in organic coatings can significantly decrease bacterial adhesion and viability with time, in comparison to the organic coating alone. The initial Ag release though at concentrations lower than the bactericidal, significantly increased *icaA* gene expression for the bacteria interacting with the Ag containing coating two hours post adhesion, especially under the higher shear rate. Stress-inducing conditions such as sub-bactericidal concentrations of Ag and high shear rate can therefore increase *icaA* expression, indicating that analysis of gene expression can not only refine our knowledge of bacterial-material interactions, but also yield novel biomarkers for potential use in assessing biomaterials antimicrobial performance.

**Keywords:** Bacterial Adhesion; Silver; Nanocomposite; Plasma Polymerisation; Gene Expression; Shear

## 1. Introduction

Along with the implantation of artificial organs and medical devices, the use of synthetic materials has become an indispensable part in almost all fields in medicine. Poly (ethylene terephthalate) (PET) is used in certain medical implants such as artificial heart valve sewing rings and artificial blood vessels due to its good mechanical properties and relatively high biocompatibility [1]. However, as is the case of most biomaterials, its long-term use is impeded by infections [2,3]. While a variety of microorganisms are involved in medical device-associated infections, coagulase-negative staphylococci, specifically *Sta-*

*phylococcus epidermidis*, has been identified as a predominant cause of infection in the presence of a medical device [4], due to its ability to adhere to surfaces, followed by the production of slime, and the formation of biofilm [5,6]. In the case of *S. epidermidis* the accumulative phase of biofilm formation mainly depends on polysaccharide intercellular adhesin (PIA) synthesis that is encoded by the *icaADBC* operon [7,8].

Recent publications have outlined the necessity to develop a strategy to reduce bacterial adhesion to aortic grafts [9,10]. The main goals of management are the removal of the infected graft material and the re-establishment of vascular continuity [9,11]. However, these procedures are associated with a high mortality and

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re-infection rate. Moreover, conservative management comprising long-term intravenous antibiotics has been reported [12]. Although the loading of materials with antibiotics seems to be an effective method to reduce biofilm formation, the ongoing release of antibiotics promotes the development of resistant strains and the risks for the spread of such resistance, following the biomaterial prophylactic and therapeutic clinical use [13].

An alternative to antibiotics for reducing bacterial viability and adhesion on medical devices is to focus on materials that release antimicrobial agents such as Ag [14]. It has been suggested that impregnation of Ag into a coating can be more effective than direct surface coating alone, since surface Ag can be readily deactivated by protein anions, while Ag release cannot be controlled in the case of Ag coatings [15,16]. Polymers that release Ag in the oxidised form have shown strong antibacterial activity and would act as reservoirs of Ag and be capable of releasing Ag for extended periods [17].

In this direction the present study investigated the effectiveness of Ag-containing organic coatings (Ag: CxHyOz) plasma-deposited on polyethylene terephthalate (PET), which contains oxygen functionalities that can be involved in the mechanism of Ag release, against initial-bacterial adhesion and biofilm formation and how this is related to *icaA* gene expression.

Assessment of *icaA* DBC operon genes' expression has become crucial to the understanding of the pathogenesis of *S. epidermidis* infections [18,19], and reverse transcription (RT) followed by polymerase chain reaction (RT-PCR) represents a powerful tool for gene expression studies through the detection and quantification of mRNA. Quantification is realized by the relative RT-PCR by the determination of the expression level of the target gene versus a housekeeping gene [20]. The *icaA* gene product is a transmembrane protein with homology to N-acetyl-glucosaminyltransferase [21]. The *icaA* gene was therefore selected to be tested as marker of *ica* operon activity, because it encodes the first component of biofilm formation.

Although there is evidence that Ag interferes with the bacteria-bacteria interactions [22], according to our knowledge, there is no publication so far relating the effect of Ag on *icaA* expression.

Moreover, since the process of bacterial adhesion to indwelling medical devices is associated in most cases with flow of body fluids [23], in this work bacterial adhesion to the various substrates was examined under two shear rates 50 and 2000 s<sup>-1</sup>, which corresponded to the physiological shear rates for stable laminar flow in blood vessels, and the effect of shear rate on *icaA* expression was investigated as well.

## 2. Materials and Methods

### 2.1. CxHyOz and Ag:CxHyOz Coatings Deposition

Organic coatings containing Ag were deposited on PET substrates through a combined strategy in which a plasma enhanced chemical vapour deposition process is simultaneous with a sputtering ones. A flow of Diethylene glycol dimethyl ether vapours of 0.25 sccm mixed with 20 sccm of argon was used as gas feed. An asymmetrical, parallel plate plasma reactor described in detail elsewhere was used for the plasma depositions [14]. Briefly, the reactor is equipped with a 7 cm dia. RF electrode and a ground one of 18 cm separated each other by a gap of 6 cm. Due to the smaller area of the cathode, under proper experimental conditions (high RF power, low pressure, low DEGDM/Ar flow ratio), a bias-induced ion bombardment results at this electrode, which prevents the deposition of any coating, and induces the sputtering of silver atoms, as evidenced by Optical Emission Spectroscopy [24,25]. Substrates were positioned at the large, ground electrode. The chemical composition of Ag:CxHyOz films (C/O/H composition, Ag-content), as well as the dimensions of the Ag clusters can be tuned by adjusting the deposition parameters. A pressure of 50 mTorr and a power of 60 W were used for the plasma depositions.

In order to investigate if the nanocomposite containing coating Ag or the organic one is responsible for the antibacterial character of the material, plasma deposition of the organic coating was realized under the same experimental conditions by replacing the silver target with a stainless steel one.

### 2.2. Physicochemical Characterization of the Coatings

The plasma deposited coatings were analyzed by means of physicochemical characterizations performed using X-ray Photoelectron Spectroscopy (XPS), Water Contact Angle (WCA) measurements, Atomic Force Microscopy (AFM) and Ag release measurements. The aim of such analysis was to assess if the chemical composition of the organic matrix (*i.e.* CxHyOz) was the same with or without the presence of Ag embedded in the coating, to assess if the chemical/morphological changes derived from the inclusion of Ag in the coating can dramatically affect the wettability of the analyzed samples, to investigate if there was any change in the surface morphology that can be attributed to the presence of Ag clusters included in the organic layer during plasma process and to evaluate the amount of Ag that was released from the substrate within the first 24 h.

### 2.2.1. X-Ray Photoelectron Spectroscopy

(XPS) measurements were performed with a Theta Probe Thermo VG Scientific instrument (base pressure  $1 \times 10^{-9}$  mbar) equipped with a monochromatic AlK $\alpha$  radiation ( $h\nu$ : 1486.6 eV) operating at 300 W with a spot size of 400  $\mu\text{m}$ . The analysis was carried out by an angle resolved mode acquiring simultaneously information from different take-off angles (T.O.A.s) ranging from  $59.5^\circ$  to  $14.5^\circ$  with a conical angle of acceptance of  $33^\circ$ , by using a two-dimensional detector that collects signal intensities in both photoelectron energy and photoemission angle. The two-dimensional detector was placed at the output plane which has the photoelectron energy dispersed in one direction and the angular distribution dispersed in the other direction. Samples were neutralized for the electrostatic charging by means of a flood gun (Mod. 822-06 FG) operating at 400  $\mu\text{A}$  emission current, 40 V extraction voltage at  $2 \times 10^{-7}$  mbar to correct differential or non-uniform charging. The high resolution spectra were shifted to their correct position by taking C1s spectrum centred at 285.0 eV as reference [26].

### 2.2.2. Water Contact Angle Measurements

A Ramè-Hart-NRL mod. 100 was used to measure dynamic water contact angle (WCA) values with double distilled water. Advancing WCA ( $\theta_{\text{adv}}$ ) was measured by increasing the volume of the water drop in 2  $\mu\text{l}$  steps, until the WCA remained constant. Receding WCA ( $\theta_{\text{rec}}$ ) was measured by decreasing the volume of the drop until the WCA value remained constant and the solid/liquid interface started to decrease. Five samples from each group were utilized for WCA measurements. For each sample, five readings of  $\theta_{\text{adv}}$  and  $\theta_{\text{rec}}$  were measured at different regions and averaged.

### 2.2.3. Atomic Force Microscopy (AFM)

Topographic AFM images were collected in tapping mode by using a PSIA XE-100 SPM System operating in air and at room temperature. A high frequency silicon cantilever for non-contact/tapping mode purchased from NanoWorld was used. A silicon SPM sensor for non-contact AFM (Park Systems) having a spring constant of  $42 \text{ N}\cdot\text{m}^{-1}$  and a resonance frequency of 330 kHz was used. Micrographs were collected on six areas of each sample, with a scan size area of  $5 \times 5 \mu\text{m}^2$ , by sampling the surface at a scan rate within 1.0 - 0.5 Hz and a resolution of  $256 \times 256$  pixels. Topography AFM images were processed by using the XEI software to obtain statistical data of the surface root-mean-squared (RMS) roughness values of the films and mean heights of sample features.

### 2.2.4. Silver Release

The amount of Ag release was evaluated by Inductively

Coupled Plasma Mass Spectrometry (ICP-MS) analysis which was performed by means of a TJA-IRIS-Advantage spectrometer (laboratory CHIMIE s.r.l. Triggiano, Bari) with echelle optics and Charge Injection Device (CID) semiconductor detector, axial and radial viewing and a wavelength range 170 - 900 nm. Plasma coated samples were soaked in 50ml of double distilled water at  $37^\circ\text{C}$  for 24 hours and the liquid was analyzed by the ICP-MS.

### 2.3. Bacterial Culture Conditions

The bacterial strain used in this study was the *ica*-positive slime producing reference *S. epidermidis* ATCC 35984. Bacteria that were in the mid-exponential growth phase, after growth in Brain-Heart Infusion Broth (BHIB, Difco Laboratories, Detroit, USA), were harvested and resuspended in 0.9% NaCl at a concentration of  $3 \times 10^9$  Colony Forming Units (CFUs)/ml [27].

### 2.4. Dynamic Bacterial Adhesion Assays

To evaluate the bacterial adhesion under flow conditions and investigate the combined effect of flow and surface chemistry on *ica* gene expression, the parallel plate flow chamber (PPFC) described by Stavridi *et al.* [28], was used. The pump was programmed to travel the pistons back and forth every 60 s. The shear rate ( $S$ ) was calculated by the following formula:

$$S = \frac{6Q}{Wh^2} \quad (1)$$

where  $Q$  is the flow rate,  $W$  (width of the chamber) = 0.015 m and  $h$  (height of the chamber) =  $0.35 \times 10^{-3}$  m.

All experiments were carried out at  $37^\circ\text{C}$  by placing the experimental setup inside a thermostated box (INFORS HT, Bottmingen, Switzerland), as described by Foka *et al.*, [27]. Two shear rates were used: 50 and 2000  $\text{s}^{-1}$  and bacterial adhesion and gene expression were examined two hours post adhesion.

### 2.5. Examination and Quantification of Bacterial Adhesion and Biofilm Formation

#### 2.5.1. Colony Forming Units Counting Method

After the adhesion experiments, adherent bacteria were detached by immersing each sample in trypsin (Sigma-Aldrich) at  $37^\circ\text{C}$  for 5 min and using a cell scraper (Sigma-Aldrich). Trypsin was inactivated with Fetal Bovine Serum (FBS, Sigma-Aldrich). Then 10-fold serial dilutions of the detached adherent bacteria were inoculated onto Trypticase Soy Agar (TSA, Difco Laboratories, Detroit, USA) plates, and the numbers of adherent bacterial colonies were counted after 18 h of incubation at  $37^\circ\text{C}$ .

### 2.5.2. Scanning Electron Microscopy

In order to be examined with Scanning Electron Microscope (SEM)(JEOL-JSM 6300, Hertfordshire, UK), each sample was fixed, dehydrated and sputter coated with gold. Images were processed using the Image Pro Plus Analysis Software (Media Cybernetics), in order to quantify the percentage of surface area covered by bacteria.

### 2.5.3. *icaA* Gene Expression Study

After the adhesion experiments, adherent bacteria were detached by trypsin (Sigma-Aldrich) and collected by centrifugation, as described by Foka *et al.*, [27]. The bacterial pellets, kept at  $-20^{\circ}\text{C}$ , were used for RNA isolation, within a week after collection using the Trizol method (Invitrogen, Carlsbad, USA), while the samples were examined in sets of four so that their processing was well controlled and consistent [27]. Genomic DNA contamination was tested by PCR with specific primers, using the appropriate reaction mix composition and thermal conditions for *icaA* gene [29] and a 207 bp part of the region V in the 23S rDNA gene [27]. Complementary DNA (cDNA) synthesis was carried out using 0.5 ng total RNA, 50 ng random hexamers primers and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen).

Real Time PCR for *icaA* gene and the 207 bp part of the region V in the 23S rDNA gene were carried out with the RotorGene device (RG-3000, Corbett Research, Sydney, Australia). The same primers used for the conventional PCRs were applied [27,30]. Each run included one *ica*-negative and a non template control as negative controls. The specificity of the PCR products was confirmed by analysis of the dissociation curve. The results were evaluated using the Rotor-Genie Analysis Software

6.0. The efficiency of reactions was calculated from the slope according to the equation  $E = 10^{-1/\text{slope}}$  [20].

Relative RT-PCR constituted the rate of the expression levels of *icaA* comparing the absolute values towards those of the reference gene (23S rDNA) [27,31].

## 3. Statistical Analysis

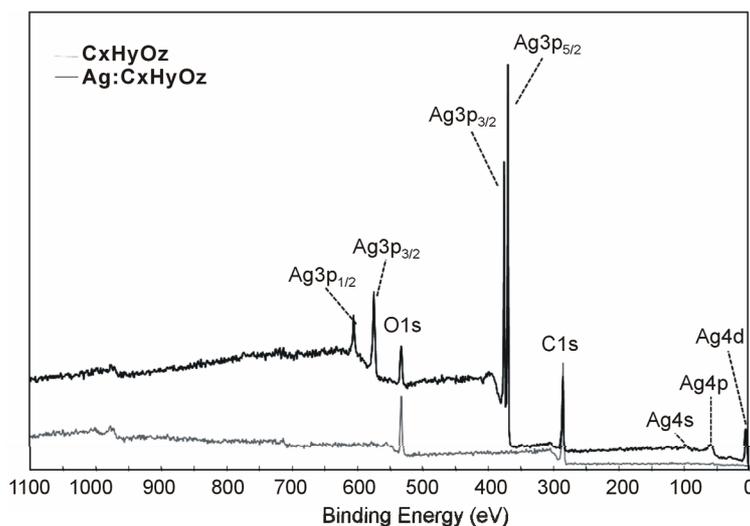
The effects of the surface chemistry and flow conditions on bacterial adhesion, biofilm formation, and *icaA* gene-expression were statistically analyzed using the SPSS package for windows. One way analysis of variance (ANOVA) was performed using the Scheffe significant difference test. Moreover, regression analysis and correlation coefficients ( $R^2$ ) were obtained using SPSS. Correlations were taken as significant for  $p < 0.01$ .

## 4. Results

### 4.1. X-Ray Photoelectron Spectroscopy

**Figure 1** shows the low resolution XPS spectra acquired on CxHyOz coatings deposited in the same experimental conditions but with (*i.e.* Ag:CxHyOz) or without (*i.e.* CxHyOz) the presence of Ag embedded inside the organic matrix. When a stainless steel target was used instead of an Ag one a presence of a coating containing no other elements than carbon and oxygen is deposited as confirmed by the XPS spectrum acquired on CxHyOz sample (**Figure 1**). By replacing the stainless steel target with a Ag one, a coating containing Ag was obtained as shown by the presence of the characteristic Ag3d, Ag3p, Ag4p, Ag4s and Ag4d peaks in the low resolution spectrum (see Ag:CxHyOz spectrum in **Figure 1**).

The best fitting of the XPS C1s spectrum acquired on Ag:CxHyOz coatings (**Figure 2(a)**) shows the presence



**Figure 1.** Low resolution XPS spectra acquired on CxHyOz and Ag:CxHyOz samples.

of different oxygen containing functionalities. The percentage of hydroxyl/ether groups attesting for the retention of the chemical structure of the precursor (*i.e.* DEGDME) in the coating was very low. **Table 1** presents the elemental percentages of C, O and Ag of the C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> and Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> coatings. The C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> coatings presented an O/C ratio 0.36, while the O/C for DEGDME is 0.5. These results indicate that the experimental conditions (*i.e.* high power low amount of precursor flow and low pressure) used for the plasma processes contributed to a certain fragmentation of the precursor during the discharge and a certain ion bombardment of the organic deposited film during the process that produces a high cross linking of the deposited film (*i.e.* high hydrocarbon content). In the case of the Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> coating the O/C ratio was 0.29 and the percentage of Ag into the nanocomposite reached almost 20%.

To assess if the organic matrix is the same with or without the presence of Ag clusters, an overlapping of high resolution XPS C1s spectra is reported in **Figure 2(b)**. It is possible to see that the C1s spectra are almost identical confirming that the presence of Ag did not change the chemical characteristics of the organic layer and that the coating C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> can be used as reference of Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> ones during antibacterial tests to disentangle the effect of the organic matrix to that one of silver.

## 4.2. Water Contact Angle Measurements

The hydrophilic character of plasma deposited coatings dramatically changed when Ag was introduced. As shown in **Table 1** the presence of Ag clusters in the coating increased the advancing water contact angle ( $\theta_{adv}$ ) from  $68 \pm 3^\circ$  in the case of the C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> coating to  $104 \pm 7^\circ$  in the case of the Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> one. Contemporary a very evident decrease of receding water contact angle ( $\theta_{rec}$ , **Table 1**) was observed for the Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> sam-

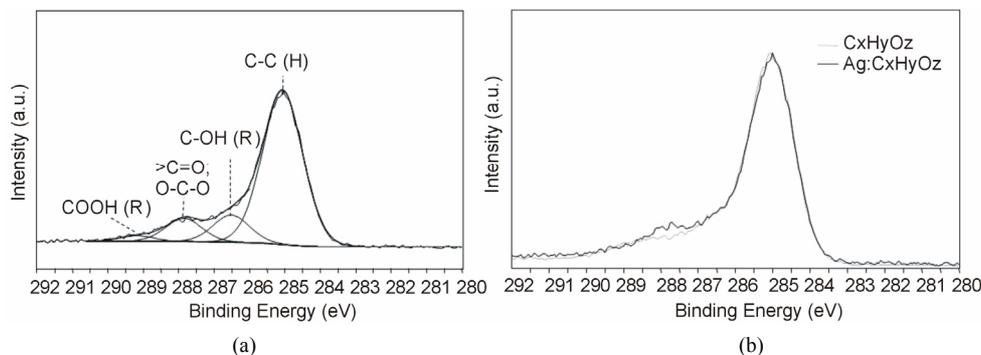
ples. These findings show that a very important hysteresis was associated to the presence of Ag clusters. Hysteresis is defined as the difference between the maximum and the minimum WCA values measured in a dynamic mode onto a surface, *i.e.* the maximum angle while the droplet is increasing its volume ( $\theta_{adv}$ ) and the minimum just before the reduction of the contact area with the solid during the volume decreasing ( $\theta_{rec}$ ). The hysteresis is originated by the presence of heterogeneities (in topography and/or chemical composition) which induce fluctuations in surface tension [32]. Since hysteresis originates from defects, the high hysteresis registered on Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> samples can be attributed to certain surface roughness other than chemical heterogeneity.

## 4.3. Atomic Force Microscopy (AFM)

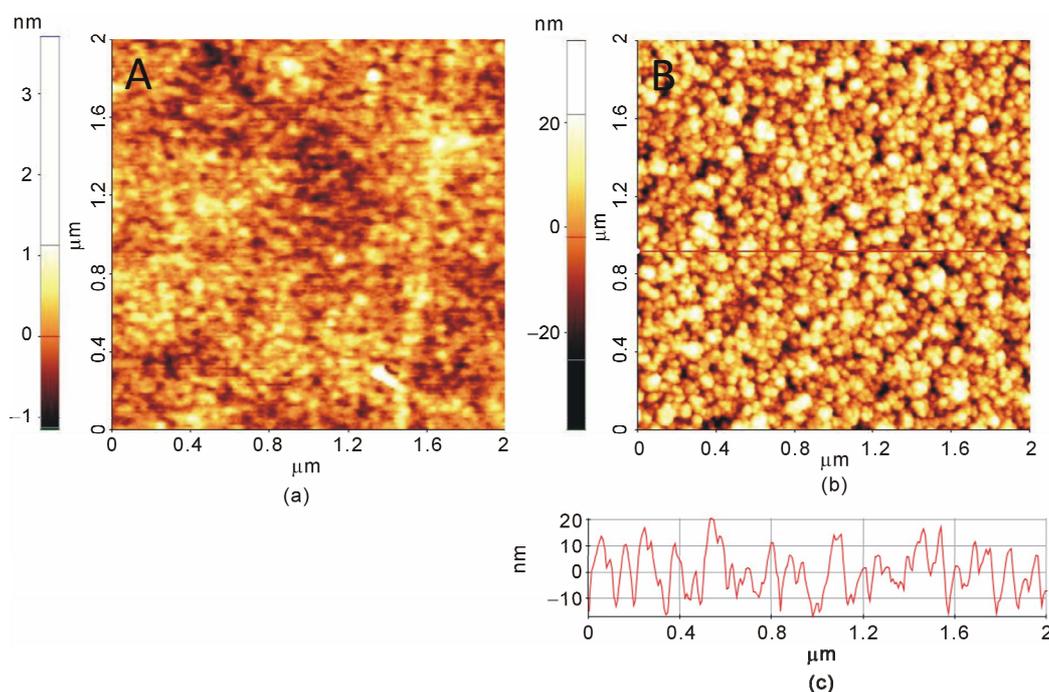
Atomic Force Microscopy (AFM) was employed to investigate the morphology of the Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> samples and further explore the hysteresis observed in the WCA measurements. **Figure 3** reports the comparison of the topography of the C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> coating with that of the Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> one. The image shows that the C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> coating appeared quite smooth with a root mean square (RMS) roughness value of  $0.2 \pm 0.1$  nm. Implementation of Ag into the organic coating significantly changed the morphology of the coating. Indeed, the RMS value increased up to ca.  $7.43 \pm 0.37$  nm and the surface was characterized by a dense and highly interconnected network of round-shape features having a mean height of  $71.3 \pm 4.5$  nm accounted for by Ag clusters.

**Table 1.** XPS chemical composition of the plasma deposited coatings and results of water contact angle measurements.

Samples	%C	%O	%Ag	$\theta_{adv}$ (°)	$\theta_{rec}$ (°)	$\theta_{stat}$ (°)
C <sub>x</sub> H <sub>y</sub> O <sub>z</sub>	66.1	24.0	---	$68 \pm 3$	$52 \pm 3$	$61 \pm 1$
Ag:C <sub>x</sub> H <sub>y</sub> O <sub>z</sub>	62.4	17.9	19.7	$104 \pm 7$	$2 \pm 7$	$108 \pm 2$



**Figure 2.** (a) XPS C1s spectrum acquired on Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> sample and best fitted in four components centred at  $285.0 \pm 0.2$  eV (alkyl, C-C; C-H),  $286.5 \pm 0.2$  eV (hydroxyl/ether, C-OH/COR),  $287.8 \pm 0.2$  eV (carbonyl/aldehyde or Hemiacetal/Hemiketal, >C=O/O-C-O),  $289.2 \pm 0.2$  eV (carboxyl/ester, COOH/COOR); (b) overlapping of C1s spectra acquired on C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> and Ag:C<sub>x</sub>H<sub>y</sub>O<sub>z</sub> samples.



**Figure 3.** 2DAFM topography images of bare a) CxHyOz and b) Ag:CxHyOz films. In panel c) cross sectional line profile taken along the red line of panel B.

#### 4.4. Silver Release

ICP-MS characterization of plasma modified materials show a release of silver of  $200 \pm 20$  ppm/cm<sup>2</sup> in the first 24 hrs.

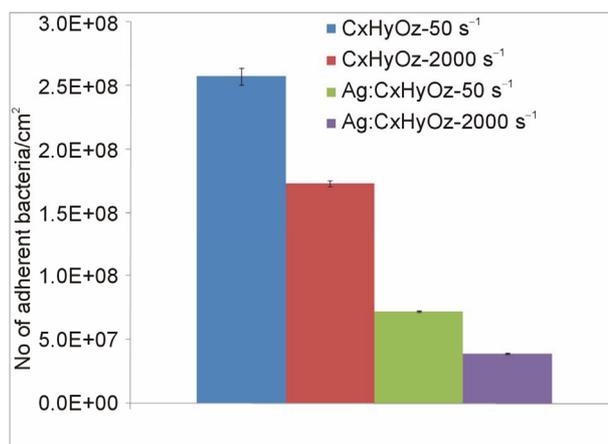
### 5. Bacterial Adhesion and Biofilm Formation

#### 5.1. Colony Forming Units Counting Method

**Figure 4** shows the combined effect of surface chemistry and shear rate on bacterial adhesion (number of adherent bacteria/cm<sup>2</sup>), as this was quantified by the Colony Forming Unit (CFU) method. Bacteria adhered significantly more to the CxHyOz coated PET in comparison to the Ag:CxHyOz coated one ( $p < 0.01$ ). A decrease in the number of adherent bacteria, for both materials, was observed when the shear rate increased from  $50 \text{ s}^{-1}$  to  $2000 \text{ s}^{-1}$ . This decrease was significantly different ( $p < 0.01$ ), for all possible combinations. The lowest number of attached bacteria was calculated under the higher shear rate onto the Ag:CxHyOz coated PET.

#### 5.2. Scanning Electron Microscopy

The SEM images revealed that, although the CxHyOz coated PET yielded the highest number of adherent bacteria, no slime production was observed while the bacteria were attached in a monolayer (**Figure 5(a)**). In the case of the Ag:CxHyOz coated PET the number of adherent bacteria was much lower than in the case of the

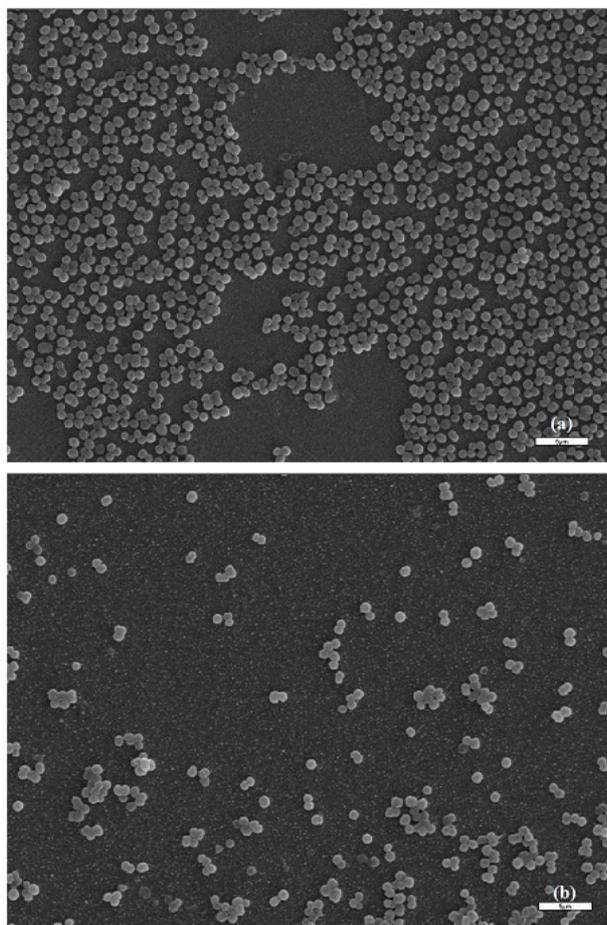


**Figure 4.** Mean values and standard deviation ( $n = 3$ ) of the number of adherent bacteria/cm<sup>2</sup> ( $N$ ) onto the CxHyOz and Ag:CxHyOz coated PET, two hours post adhesion, under shear rate of  $50 \text{ s}^{-1}$  and  $2000 \text{ s}^{-1}$ .  $p < 0.001$  for all the possible combinations.

CxHyOz coated PET, for both shear rates, however apart from isolated bacteria, small aggregates and in some cases a second layer of adherent bacteria could be observed two hours after adhesion (**Figure 5(b)**), under both shear rates. The percentage of surface coverage by bacteria is summarised in **Table 2**.

#### 5.3. *icaA* Gene Expression

Under all the tested conditions, *icaA* was mostly expressed



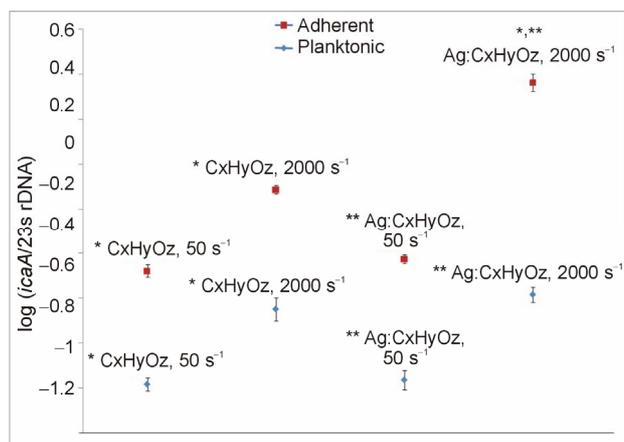
**Figure 5.** SEM images of bacteria interacting with the CxHyOz coated substrate (a) and the Ag:CxHyOz coated substrate (b) under  $50 \text{ s}^{-1}$ .

**Table 2.** Mean values and standard deviation ( $n = 3$ ) of % surface coverage of the surface by bacteria, as this was quantified using SEM and image analysis.

Samples	% Surface Coverage
CxHyOz, $50 \text{ s}^{-1}$	$72.7 \pm 2.5$
CxHyOz, $2000 \text{ s}^{-1}$	$55.9 \pm 3.1$
Ag:CxHyOz, $50 \text{ s}^{-1}$	$21.3 \pm 1.9$
Ag:CxHyOz, $2000 \text{ s}^{-1}$	$16.5 \pm 2.3$

by the adherent than the planktonic bacteria (**Figure 6**) ( $p < 0.001$ ), showing its implication to biofilm formation by the adherent bacteria.

*icaA* expression was always statistically significantly higher for bacteria interacting with the Ag:CxHyOz coated PET, both adherent and planktonic, than for those on the CxHyOz coating ( $p < 0.001$ ) (**Figure 6**). Furthermore, the increase in shear rate statistically significantly enhanced the expression of *icaA* gene for both adherent and planktonic bacteria, interacting with both CxHyOz and Ag:CxHyOz coated PET ( $p < 0.001$ ) (**Figure 6**).



**Figure 6.** Effect of shear rate and surface chemistry on adherent and planktonic bacterial *icaA* gene expression, two hours post adhesion. Results are expressed as mean values and error bars indicate standard deviation ( $n = 3$ ). \* and \*\*  $p < 0.001$ , when the results for either the adherent or the planktonic bacteria are compared.

## 6. Discussion

In this study the contributions of Ag and shear rate on *S. epidermidis* adhesion, accumulation and *icaA* expression were investigated through the plasma deposition of nanocomposite coatings with and without 20% Ag; Ag:CxHyOz and CxHyOz respectively. The relative contribution of physicochemical and hydrodynamic interactions on bacterial adhesion and specific gene expression was addressed by quantitative measurement of bacterial adhesion and gene expression on CxHyOz and Ag:CxHyOz coated PET in laminar flow, as a function of fluid shear rate.

The CFUs counting method showed that *S. epidermidis* adhered significantly more to the CxHyOz coating in comparison to the Ag:CxHyO zone ( $p < 0.01$ ), and this is in agreement with our previous study [33] that showed decreased bacterial adhesion to the Ag plasma coated poly (vinyl chloride) (PVC), in comparison to the untreated PVC. In parallel, it was observed that the increase in shear rate significantly reduced bacterial adhesion to both materials. However, almost 30% of the attached to the Ag:CxHyOz coating bacteria, in comparison to the CxHyOz, remained alive and although the number of adherent bacteria was significantly lower than in the case of the CxHyOz coated PET, apart from isolated bacteria, small aggregates and in some cases a second layer of adherent bacteria could be observed on the Ag:CxHyOz coated PET, under both shear rates.

Although Chaw *et al.*, [22] and Qu *et al.*, [34] AFM studies showed that Ag ions interfere with bacteria-bacteria and bacteria-material interactions, through the binding of Ag ions to electron donor groups of the biological

molecules, leading to reductions in the number of binding sites for hydrogen bonds and electrostatic and hydrophobic interactions and, hence, the destabilization of the biofilm structure [22,34]. Qu *et al.*, showed that far fewer *S. aureus* and *S. marcescens* than *P. aeruginosa* were actually killed by the Ag ions release and this was attributed to the differences in the cell wall [34]. Specifically, the cell walls of Gram-positive species contain 3 - 20 times more peptidoglycan than Gram-negative bacteria [35], and probably bind some portion of the Ag ions. Consequently, Gram-positive bacteria are generally less susceptible to antibacterial agents containing Ag than Gram-negative species [35]. This is in agreement with Sandström observation that when a staphylococcal suspension of  $10^8$  -  $10^9$  CFU/ml was used, the minimum bactericidal concentration of Ag was found to be above 987 ppm [36]. This could explain our present results that showed that almost 30% of the attached to the Ag:CxHyOz coating bacteria, in comparison to the CxHyOz coating, were alive, 2 hours after adhesion and while 100 ppm of Ag had been released.

In order to investigate possible links between phenotypic responses and genetic activity of bacteria during bacteria-material interactions under shear conditions, assessment of *icaA* gene expression towards a part of 23S rDNA was performed, for both planktonic and adherent bacteria. *icaA* expression seems to be influenced by both the material surface chemistry and the shear conditions mainly for the adherent bacteria at early adhesion stages. In particular, it was observed that *icaA* expression level was always higher for the adherent bacteria in comparison to the planktonic, showing its implication to biofilm formation by the adherent bacteria and not that much by those that are in suspension.

In addition, the results showed that *icaA* gene expression was influenced by the surface chemistry as well, mainly for the adherent bacteria. In particular, higher expression of *icaA* gene was observed for the bacteria that were interacting with the Ag:CxHyOz coating than with the CxHyOz one, for all the tested conditions. This possibly means that although the Ag:CxHyOz coating reduced bacterial adhesion, in comparison to the CxHyOz one, the amount of Ag that was released and did not kill all the adherent bacteria caused increased expression of *icaA* gene. In the case of the planktonic bacteria, the *icaA* gene expression was not as much influenced by the material surface chemistry, as in the case of adherent bacteria.

Apart from the stress caused to bacteria by the sub-bactericidal concentration of Ag and resulted in enhanced *icaA* gene expression, the increase in shear rate significantly increased the expression levels of *icaA* as well, for both CxHyOz and Ag:CxHyOz coatings and for both adherent and planktonic bacteria, meaning that the sur-

face chemistry in combination with the shear conditions affect the phenotypic responses of bacteria to bacteria-material and result in gene expression profile alterations.

Trying to compare these results with other in the literature, there are some studies that have also identified genes that were differentially expressed in biofilm and planktonic populations. In particular, Prigent-Combaret *et al.*, reported that the colonic acid exopolysaccharide (*wca* locus) was expressed in higher levels by adherent *E. coli* bacteria than by the planktonic, whereas the flagellin (*fliC*) gene expression was reduced in biofilms [37]. Furthermore, Becker *et al.*, identified five genes which were differentially expressed in biofilm and planktonic populations of *S. aureus* [38], while early high transcription of *icaA* has been also reported in a study using stainless steel [39] and during an *in vitro* foreign-body colonization model [40]. Concerning the effect of substances' sub-bactericidal concentrations on gene expression Fitzpatrick *et al.*, have also shown that the number of *ica*-positive biofilm-positive bacteria under stress-inducing growth conditions, and in particular in the presence of NaCl, ethanol or subinhibitory tetracycline concentrations, increased significantly from 15.3% to 73%, in comparison to the standard growth conditions [41]. Moreover, Kuehl *et al.*, observed biofilm enhancement by furanone in staphylococci at subinhibitory concentrations, which was correlated with an increase in PIA synthesis and dependent on *luxS* gene expression, although the mechanism of interaction between *luxS* and PIA under furanone treatment remained unclear [42]. In another study though, the level of biomaterial-induced *icaA* expression could not be correlated with the material physicochemical properties or the amount of biofilm formed, but with the ability of bacteria in surviving antibiotic attack [43]. These results therefore showed that there was an increase in biofilm formation when bacteria were under stress due to various environmental factors. Alterations of these factors can ultimately give an insight of how bacteria-material interactions are influenced by their surroundings.

In our previous study it was also observed that bacterial adhesion as well as slime production and biofilm formation were influenced by the surface chemistry [27]. In particular, much higher biofilm formation was observed on the hydrophobic CH<sub>3</sub>-terminated glass than on the hydrophilic OH-terminated one, for three *S. epidermidis* strains, and this was in agreement with *icaA* and *icaD* increased gene expression for the bacteria adhering to the CH<sub>3</sub>-terminated substrate. The decreased surface energy of the Ag:CxHyOz coating, in comparison to the CxHyOz, could be therefore another reason for the increased *icaA* gene expression in the case of the

Ag:CxHyOz coating two hours post adhesion. Furthermore both this study and the previous one [27] showed that the flow conditions and the associated hydrodynamic forces strongly influence not only the number of attached bacteria, but genes functionality related to biofilm formation as well.

In the same directions, Thomas *et al.*, observed that shear stress strengthened *E. coli* attachment to red blood cells through biological interactions that were enhanced by mechanical force pulling a ligand-receptor complex apart, and are known as “catch bonds” [44]. Moreover, Weaver *et al.*, showed that fluid flow induced biofilm formation in *S. epidermidis* PIA-positive clinical isolates [45].

These findings suggest that bacteria are able to sense and respond to surfaces and environmental signals using mechanisms that remain poorly understood and that the regulation of biofilm formation under altered growth conditions, which may exist in the *in vivo* environment, also plays a possible role in the pathogenesis of biomaterial-related *S. epidermidis* infections.

The results of the present study therefore suggest that although the incorporation of Ag in CxHyOz as a biomaterial coating can decrease bacterial adhesion and viability, in comparison to the CxHyOz alone, its presence at sub-bactericidal concentrations, especially when Gram-positive bacteria are implicated, and its application at high shear rate environments such as aortic grafts and catheters, can significantly increase *icaA* gene expression and therefore the risk of biofilm formation. In this direction, the effect of shear stress on alterations in genes expression of adhering bacteria is another parameter that should be taken into consideration for the design of biomaterials, and the biomaterial coating strategies should be evaluated and applied after considering important details unique or specific to each application. This could probably mean that Ag release should be quicker for biomaterials that are inserted in high shear environments and when Gram-positive bacteria are implicated. Furthermore, for applications that tissue integration is desirable the use of medical devices containing Ag must be undertaken with caution, since a concentration-dependent toxicity has been demonstrated [46].

A limitation of the present study is that the reactivity of other genes contributing to *S. epidermidis* adhesion and biofilm formation under the same conditions has not been tested.

## 7. Conclusion

This study was conducted utilizing two shear rates and two surface functionalities, in order to investigate how the combination of antimicrobial properties, through plasma deposition of Ag containing nanocomposite coat-

ing, and hydrodynamic conditions influence bacterial viability, adhesion and *icaA* gene expression. The results showed that both the surface chemistry and the shear conditions parameters significantly influenced *S. epidermidis* adhesion and the expression of *ica* locus. The combination of both conventional phenotypic analysis, using the CFU method and SEM, and genotypic analysis, using the relative reverse transcription polymerase chain reaction (RT-PCR), offered the potential to explore the links between phenotypic responses to bacteria-material interactions and gene expression profile alterations, under dynamic conditions. Bacterial adhesion was much higher on the CxHyOz coated PET than on the Ag:CxHyOz coated one. However, the Ag release at sub-bactericidal concentrations in combination with the increase in shear rate significantly enhanced *icaA* gene expression for the bacteria that were attached to the Ag:CxHyOz coating. These observations indicate therefore that a quicker Ag release could possibly prevent bacterial adhesion and *icaA* gene expression, while the analysis of gene expression, along with the use of appropriate microscopies seems to be able to provide a more comprehensive and integrated insight into the bacterial-material interactions, and also introduce novel bioassays towards biomaterials antimicrobial assessment.

## 8. Acknowledgements

This project was partially funded by the programme: INTERREG IIIA: GREECE—ITALY “Establishment of a Multidisciplinary Scientific Network for the development and application of Advanced Biomaterials” 2006-2008 and by the funding of the National Staphylococcal Reference Laboratory, Greece, under the scientific responsibility of I. S. (grant C954, KEELPNO).

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