

First steps of biofilm formation on stainless steels in natural seawater

The *BASIS* Group (Biofilm Adhesion on Substrates Immersed in Sea water) :

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Numerous works are reported in literature concerning the biofilm growth on surfaces in seawater but the understanding of the adhesion mechanisms in the first steps of immersion is still subject to investigations. A knowledge of the chemical composition and of the kinetics of conditioning layer formation is a central requirement for a sound scientific understanding of biofilm growth. Due to its complexity and to the interdisciplinary nature of the subject, this research asks for the collaboration between the fields of material science, surface analyses, chemistry, marine microbiology and biochemistry.

The first steps of biofilm formation on 316L stainless steels immersed in natural sea water have been studied, using a broad range of surface characterisation techniques : X-ray Photoelectron Spectroscopy (AR-XPS) for chemical characterisation of the passive film and of the adsorbed organic species, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) for chemical characterisation of the adsorbed organic species, Surface Infra-Red Spectroscopy (IRRAS) for the description of the chemical links, Atomic Force Microscopy (AFM) for the identification of the morphology, thickness and homogeneity of the organic film, and liquid drop contact-angle measurements for the determination of the hydrophilic/hydrophobic and acido-basic character of the outer surface.

Introduction :

Marine fouling of structures and equipment is a major problem for industrial and scientific activities in the sea. Costly countermeasures are needed to avoid the adhesion of such micro and macro-organisms and local pollution results from extensive use of inappropriate antifouling techniques, which have detrimental effects on the environment and are harmful to living marine resources. Moreover, some micro-organisms present a remarkable degree of tolerance to high levels of toxic molecules or heavy metals.

To cure the problem from the root it appears essential to understand the process of the biofilm growth, especially during the first steps of the biofilm settlement. An improved knowledge of the biofilm adhesion mechanisms using advanced analysis methods, is necessary to develop an alternative approach to the antifouling agents such as the implementation of new surface treatments against biofilm growth.

In this paper a particular emphasis is given on the first steps of biofilm formation on AISI 316L stainless steels immersed for short periods of exposure in natural sea water (2, 5, 8 and 24h). This was done using a broad range of surface characterisation techniques : X-ray Photoelectron Spectroscopy (AR-XPS) for chemical characterisation of the passive film and of the adsorbed organic species, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) for chemical characterisation of the adsorbed organic species, Infra-Red Spectroscopy (IRRAS) for the determination of the nature of the chemical bonds involved in the adhesion process, Atomic Force Microscopy (AFM) for the identification of the morphology, thickness and homogeneity of the organic film, and finally liquid drop contact-angle measurements for the determination of the hydrophilic/hydrophobic and acido-basic character of the outer surface.

Experimental :

The experimental details of AR-XPS, IRRAS, AFM and liquid drop contact-angle techniques have been published elsewhere (Walls *et al.* 1998). The ratios of the IRRAS processed sample spectra to an IRRAS processed background spectrum were converted to absorbance spectra. The background spectrum is recorded on a sample immersed a few seconds in distilled water and dried. The surface-energy measurements of all samples are compared with control specimen obtained after rinsing in distilled water. Positive and negative ToF-SIMS measurements were performed with a ToF-SIMS spectrometer from Phi Evans (Bertrand and Weng. 1996, Schueler *et al.* 1990, Schueler 1992). In these experiments, the sample was bombarded with pulsed primary $^{69}\text{Ga}^+$ ions (15 keV). The secondary ions were accelerated to ± 3 keV by applying a bias on the sample. The spreading of the initial energies of the secondary ions is compensated by deflection in three electrostatic analyzers. In order to increase the detection efficiency of high-mass ions, a post-acceleration of 10 keV was applied at the entry of the detector. The analyzed area was a square of 130 micron x 130 micron. With a data acquisition time of 5 min, the total dose was about 1.10^{12} ions/cm², which ensured static conditions (Briggs and Hearn 1986). The best mass resolution obtained with this ToF-SIMS spectrometer is $m/\Delta m = 11000$ at $m/z=28$ on a Si wafer. For the present samples and analytical conditions, the mass resolution was about 5000 at $m/z=29$. This highly facilitates the assignment of ions having the same nominal mass but different compositions, e.g. hydrocarbon, oxygenated, nitrogen-containing fragments, etc.

Samples preparation

1cm or 5mm square samples of AISI 316L stainless steel plate were ground with SiC emery paper (final stage 1200 grit) then with diamond paste to 1 μm and cleaned in ultra-sonic baths between each grade. Two different techniques of surface preparation were used : a rinse with acetone and

ethanol or a rinse with hexane. The samples were then washed in three successive demineralised water baths at 50 °C washed again with ethanol, and finally air-dried.

All experimental conditions concerning immersion and transport are described in (Walls *et al.* 1998). Some samples were immersed directly in natural sea water and immediately cooled. These experiments are referred to as "0 hour immersion".

Results :

Despite the surface preparation of the sample, an organic contamination layer composed mainly of hydrocarbons and of polydimethylsiloxane: PDMS, - (Si (CH₃)₂- O)_n has been detected by ToF-SIMS analyses. In that, the chemical characterisation of the adsorbed organic molecules is thus particularly difficult. PDMS is a very common surface contaminant owing to its very low surface free energy.

The presence of the conditioning film has been detected for short periods of immersion in natural seawater. As a function of immersion time, an increasing surface concentration of compounds containing C-O, C=O and O-C=O bonds is clearly observed with XPS, IRRAS and ToF-SIMS measurements. Figure 1 shows the spectra of the C1s regions recorded on samples immersed from 0 to 24 hours in natural seawater. The same behaviour is observed on the infrared spectroscopy spectra, (Figure 2), with an increase in the bands at 1050-1160 cm⁻¹ ascribed to C-O and C-O-C bonds, from 5 to 24 hours of immersion. These results are confirmed with the ToF-SIMS analyses. Table 1 shows the evolution of the relative intensities of secondary ions peaks, (CH₃O⁻, C₂O⁻) as a function of immersion time; these values are estimated using Equation 1 where *I*_{total} and $\sum I_{contam}$ are respectively the sum of relative intensities of all secondary ions and the sum of relative intensities of all specific peaks of PDMS while *I*_x is the relative intensity of the peak x.

$$I_{rel} = \frac{I_x}{I_{total} - \sum I_{contam}} \quad [1]$$

The XPS, IRRAS and ToF-SIMS measurements also reveal the presence of adsorbed amine groups from proteins. The intensity of the signal attributed to N-H or C-N, C=N species is undoubtedly higher with longer immersion time as shown in Figure 3 with the evolution of the N 1S XPS signal. The detection of an absorption band at 1500 cm⁻¹ on IR spectra after 5 hours of immersion in natural seawater also indicates a very rapid adsorption of amines on the surface (Figure 2). These results are confirmed by the semi-quantitative interpretation of the ToF-SIMS peaks intensity of NH, NH₄⁺, CN⁻ and CNO⁻ as a function of immersion time, Table 1.

The surface aspect of the stainless steels versus immersion time is given in Figure 4. However on both stainless steel or mica surfaces, the deposit is extremely heterogeneous and in the form of small particles. Figure 5 shows AFM images obtained on two mica surfaces after 24 hours of immersion in natural seawater at the same period of exposure. The same observations are made on stainless steel surfaces for each exposure time. Surface-energies measurements confirmed these results (Figure 6). For one sample the electron-donor component γ^- of the surface energy increases as a function of time while no change is observed for the other one.

Immersion Time (Hours)	NH	NH ₄ ⁺	CN ⁻	CNO ⁻	CH ₃ O ⁻	C ₂ O ⁻
0	0.506 ± 0.054	0.0966 ± 0.0076	4.07 ± 0.16	2.73 ± 0.073	0.300 ± 0.012	1.07 ± 0.037
2	1.47 ± 0.087	0.269 ± 0.064	12.1 ± 1.60	7.12 ± 1.20	0.485 ± 0.041	1.26 ± 0.024
5	1.33 ± 0.21	0.194 ± 0.023	8.37 ± 1.29	5.04 ± 1.16	0.482 ± 0.028	1.42 ± 0.15
8	1.86 ± 0.12	0.648 ± 0.081	25.7 ± 1.14	13.3 ± 0.66	0.704 ± 0.023	2.08 ± 0.11
24	2.19 ± 0.13	0.834 ± 0.084	28.9 ± 2.04	14.4 ± 1.12	0.768 ± 0.012	2.21 ± 0.13

Table 1: Relative intensities of some secondary ions peaks (‰)

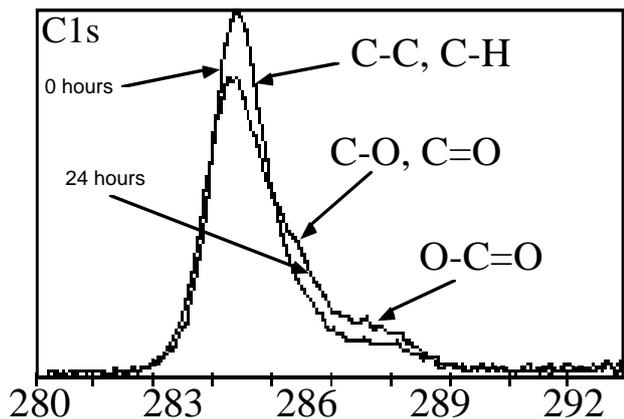


Figure 1: C1s signals after "0" and 24h immersions in natural sea water

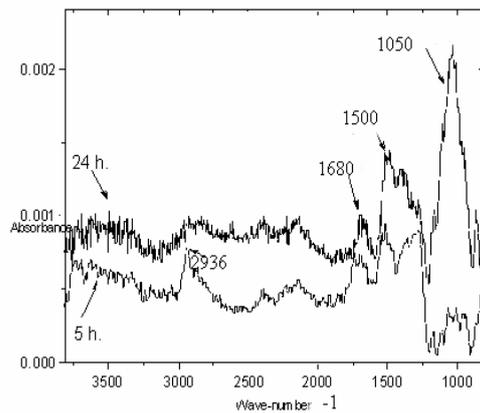


Figure 2: Infra-red spectra after 5 and 24h immersions in natural sea water

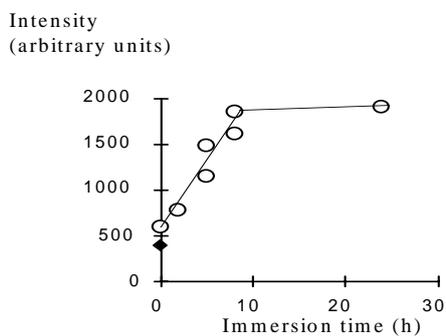


Figure 3: Evolution of the N 1s XPS signal with immersion time in natural sea water
♦ N1s polished sample, ○ N1s immersed sample

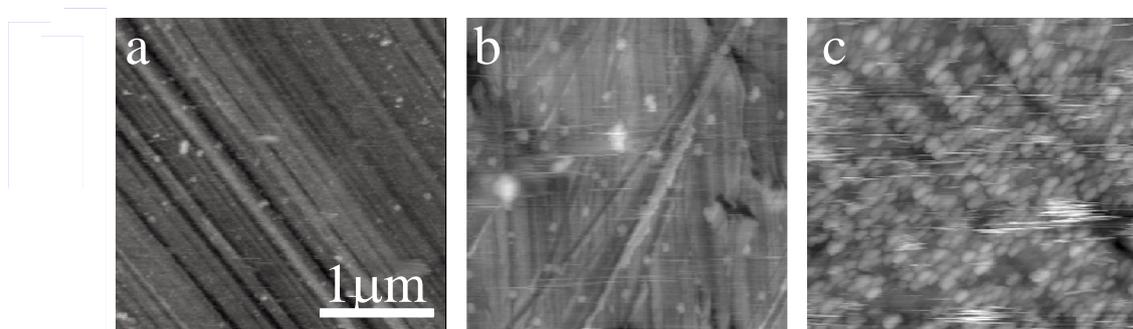


Figure 4 : Evolution of steel surface with immersion time: a = "0h", b = 5h, c = 24h. (The magnification is the same for all AFM micrographs)

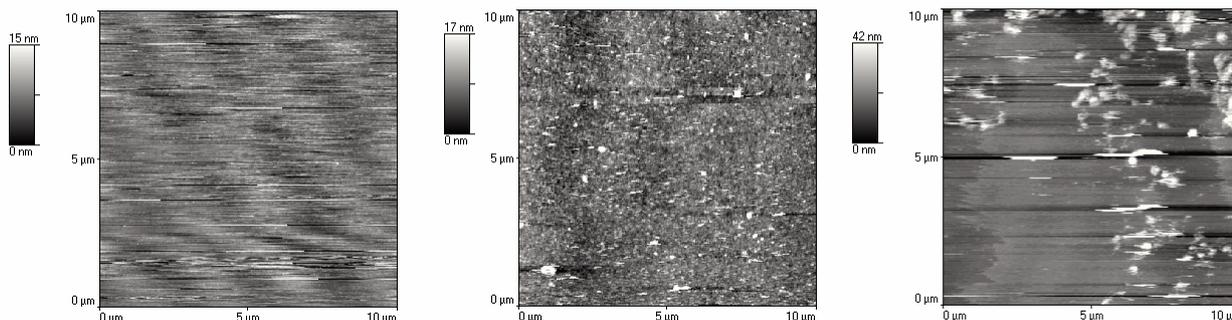


Figure 5 : Three regions on two mica surfaces exposed for 24h in nominally identical conditions

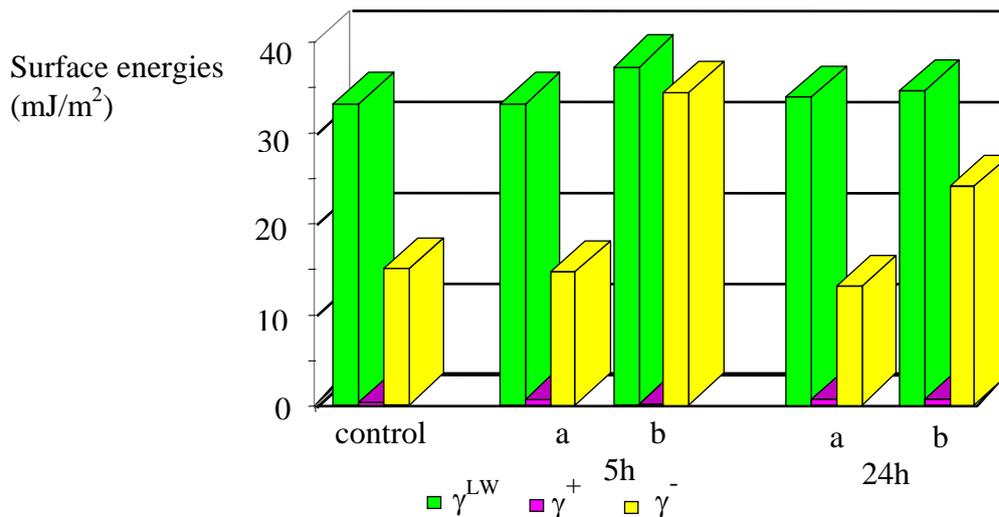


Figure 6 : Surface energies as a function of immersion time in natural sea water. The control result is after rinsing in distilled water and a and b are to two samples treated identically. γ^{LW} van der Waals, γ^+ electron-acceptor and γ^- electron-donor components of the surface energy

Conclusions

This collaboration between the fields of material science, marine chemistry, microbiology, biochemistry with advanced analytical techniques gives an insight into the chemical composition and the kinetics of conditioning layer formation. A very rapid adsorption of amines occurs on the surface followed by adsorption of carbohydrates. However the nature of the surface (stainless steel or mica) and even after 24 hours of immersion, this initial deposit is extremely heterogeneous and in the form of small particles. Further work are in progress in order to improve the cleanness of the samples before immersion in natural seawater and to characterise more precisely the early stages of bacteria adhesion and biofilm formation.

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