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***Abstract*—Silver nanocomposites represent an interesting material for biomedical applications thanks to their bacteriostatic properties. Ag-doped epoxy coatings seem a promising candidate in this field, also due to their easy production. Many are the possible techniques that enable researchers to characterize the antimicrobial behavior of materials, but most of them require specific equipment and expertise, often not present in material science laboratories. This paper describes a possible characterization which is based on a simple imaging system. Scanning Electron Microscope (SEM) micrographs are acquired in order to analyze the bacteria attachment on different samples, that is epoxy coatings containing silver nanoparticles or without their addition. The images are then processed by using a simple imaging algorithm which is based on the free $OpenCv^{TM}$ environment. The processing has been tailored to be able to work with SEM images acquired on isolating samples, which represent an additional difficulty if compared to uncoated metallic samples.**

***Index Terms*—Microbial Corrosion, Image Processing, Silver nanocomposites, Scanning Electron Microscopy**

I. Introduction

In recent years, the need for new materials able to control the proliferation of bacteria in the environment has markedly increased [1], [2]. Many are the possible solutions that have been proposed to face this important issue, and one of these is represented by organic coatings. In particular, anti-fouling paints can be classified depending on the adopted strategy to avoid the proliferation of microorganisms: non-stick fouling release coatings (having superficial properties able to avoid fouling adhesion) or coatings releasing toxic compounds [3]. In this last category, interesting solutions are represented by silver nanocomposites, in which the biocidal effect of silver ions is exploited [4].

Several solutions can be used to evaluate the antimicrobial properties of a specific material. The most common is the Agar Diffusion Test, in which the sample is placed in an agar plate inoculated with a culture of a specific microorganism. Then the antimicrobial effect is evaluated, after a period of incubation, measuring the zone of inhibition, that is the area in which the material has stopped bacterial proliferation [5], [6]. Advantages of this technique are connected to the relative easiness in which it can be implemented and to the direct assessment of the bactericidal effect. At the same time, being only a visual evaluation, a characterization of the sample surface at microscopic level is not possible as well as a quantification of microorganisms attached on it.

More sophisticated solutions, relying on optical microscopy and staining techniques, can reach a higher level of understanding about the antimicrobial properties of a specific material. In this case, three are the main possible characterizations: attachment assay, cell death essay and planktonic killing. The former test provides information about the ability of the material to avoid bacteria attachment on sample surface, without discriminating if the microorganisms are still living [7]. On the other hand, cell death essay uses staining techniques to identify bacteria on sample surface differentiating those that are still alive and those that have been killed by the bactericidal effect of the material [8]. Finally, counting of colony-forming units (CFU) provides information about the presence of viable bacteria in the solution where the material has been immersed, enabling the researchers to estimate the planktonic killing, that is the killing of microorganisms suspended in the solution [9]–[11].

Main limitation of these techniques are related to the specific expertise and equipment needed, often not available in material science laboratories. On the other hand, the Agar Diffusion Test, the simplest characterization between those presented above, can hardly be coupled with electrochemical techniques (widely used for corrosion resistance assessment), which is an important limitation as far as the material characterization is concerned [12]. Because of this, great advantage could arise from an imaging system able to analyze images acquired through electron microscopy (an instrumentation routinely used in material science laboratories), providing information about bacteria attachment on sample surface. Imaging approaches have been employed in several fields [13]–[15] mainly due to their quite limited cost [16]–[18] once the images have been acquired. Morover, another advantage is represented by the possibility of analyzing images also after long times in order to compare results of previous tests with new measurements performed as an example in different conditions.

In this work, an image processing able to assess

bacteria attachment on organic coatings is presented. Images are collected using a Field Emission Scanning Electron Microscope (FESEM), which is easily capable of providing the magnification required to examine bacteria attachment and is usually available in any material science laboratory, so that specific additional equipment or skills is usually not required. The image processing is obtained by using the open-source $OpenCV$ library, which is available for different operating systems and can also be installed on low-cost microprocessors so that it could in principle be embedded into a low cost measuring system [19], [20]. Results achievable thanks to this image processing are presented showing the data obtained from the characterization of Ag-doped epoxy coatings.

II. Image Processing by means of the $OpenCv$ environment

Bacteria identification and estimation of the relative amount of attached bacteria on a surface can be performed trying to separate in the image the areas covered by the bacteria themselves. In principle this operation is reasonably easy, though questionable, when performed manually by an operator, but it is much more difficult when performed in an automatic way. The request is, for each pixel on the image, to define if it belongs to a bacterium or not, i.e. the image, which in general is a gray-scale one, must be converted into a binary one applying some form of threshold. After that, it becomes easy to estimate the relative amount of bacteria simply by counting the pixels belonging to bacteria areas with respect to the total. The real problem is therefore in converting the image to a binary representation. Bacteria can have dimensions of few micrometers or below, therefore a high resolution microscope is required to observe the surface. In order to avoid the use of staining techniques, the authors decided to use micrographs taken by means of a Scanning Electron Microscope, which has a sufficient resolution to identify bacteria.

With this goal, to assess the amount of bacteria attached onto the samples surface, several images have been collected by using a Field Emission Scanning Electron Microscope (FESEM - Supra 40 by Zeiss). Like any Scanning Electron Microscope, images are closely correlated to the sample composition and conductivity: areas with high conductivity lead to bright areas while on the contrary areas with lower conductivity lead to dark areas on the image. This makes it easy to detect spots with low conductivity on a conductive surface, but makes it more difficult to highlight areas of different composition but similar conductivity. Actually in the case of a polymeric coating, being it non-conducting, the sample must be coated with a thin layer of a conductive material (such as Gold, Chromium or Graphite). This lead to an image in which colors are more uniform and bacteria are difficult to distinguish from the substrate. Of course, the operator can change the image contrast and brightness to adjust it for the sample, but the problem still remains. As an example, Fig. 1 shows side by side the images of two samples with the same magnification and taken by the same instrument. Both images refer to samples that have been immersed in a solution containing bacteria: the sample on the left refers to a bare metal (therefore a conductive material), while the sample on the right refers to a metal coated by epoxy resin (i.e. a non-conducting sample). The micrographs were acquired at a magnification of $5000x$ and refer to an area of about $60\ \mu\mathrm{m} \times 40\ \mu\mathrm{m}$, while the expected bacteria size is in the range of $2\ \mu\mathrm{m}$ to $10\ \mu\mathrm{m}$. The pixel resolution of the images was of about $0.065\ \mu\mathrm{m/pixel}$.

Some bacteria can be seen attached on the surface of both samples, but the images appear quite different. On the left, bacteria appear as dark sports on a light background, as the contrast is given by the different conductivity. In the other case, as the sample has been coated by a conductive thin layer of Chromium, the contrast is limited and is only given by the morphology of the surface (i.e. by microorganisms attached on the epoxy resin).

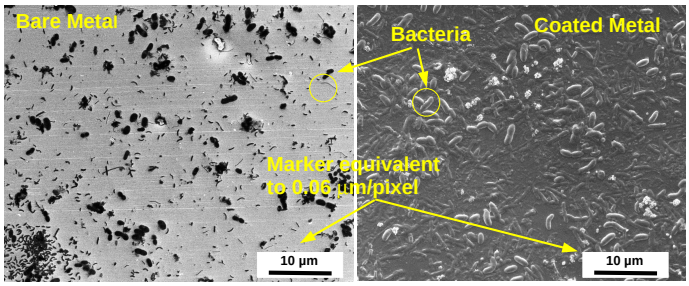

Fig. 1. FESEM micrographs of samples after immersion in a solution containing bacteria. On the left, a bare metal sample (Stainless Steel), on the right a sample coated by epoxy resin.

In addition to the intrinsic difference of the images due to the sample conductivity, also the average sample brightness can change according to the position on the sample. This can be a consequence either of a not perfect sample planarity, or of the so-called charge effect [18]. In any case a correction of this effect is reasonably easy if the spot to be identified has an high contrast [21], but can be critical if such a contrast is minimal like in the case of coated samples.

A possible solution to this problem is to avoid treating the image as a whole and compute a local brightness average value according to the point and to use this value as the reference point to decide if a pixel is a candidate to be considered as belonging to a bacteria aggregate or not.

The size of the image to be considered for the local brightness average depends on the size of the expected spots: on one hand selecting a too small area might be detrimental turning out in missing some of the spots, on the other hand a too large area may lead to missing a local brightness average change. Since with the used acquisition parameters the bacterium size is expected to be of the order of $150\ \mathrm{pixel}$ maximum, a size of $200\ \mathrm{pixels}$ was selected to be considered for the local brightness estimation.

The threshold process needs also to be adapted to the image appearance, in particular in the case of a coated samples, where the contrast between the background and the bacteria is minimal. In the case of the bare metal sample, bacteria can

be easily identified as dark spots, i.e. when their brightness (e.g. the black level) is at least 20% lower than the average brightness. In the case of the coated sample, bacteria appear slightly brighter than the background, so the threshold process tags them if their brightness is at least 2% higher the the background. Of course this encompasses most of the bacteria, but also some of the points which are not bacteria, but have a higher brightness.

Fig. 2 shows as an example the result of the threshold process on two images referring to the same samples of Fig. 1. It is clear how in the case of the uncoated sample the threshold process works easily, while in the case of the coated sample the resulting image is much more complex. In addition, the color brightness inversion due to the different conductivity is clear and in the coated sample the attached bacteria appear as white areas over a dark background.

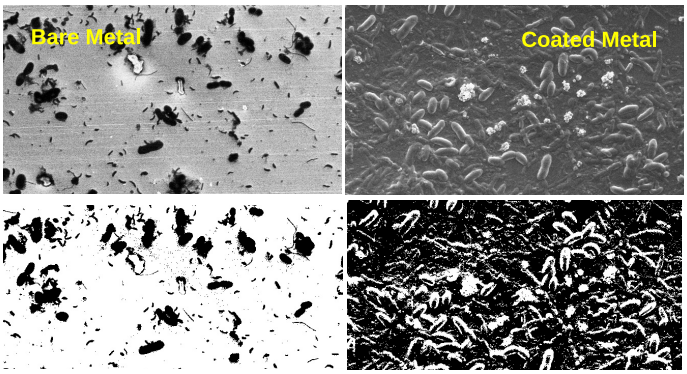

Fig. 2. Example of results of brightness estimation for the threshold.

In both cases the threshold process works by auto-adapting to the average local brightness.

III. Bacteria identification

Bacteria identification can be carried out on the images after the threshold procedure by trying to identify closed areas with dimensions compatible with the bacteria. The authors decided to perform such identification taking advantage of the OpenCV open source environment [22], [23].

The `findContour` function was used to identify bright spots. This function returns a list of contours identified by a closed path. In order to remove small spots and to avoid counting areas too thin to be considered bacteria, two parameters have been computed for each contour. The first parameter is the area A_c enclosed by the contour, defined as:

$$A_c = \sum_x \sum_y a_{ij} \tag{1}$$

where the sum extend over the entire identified area. Contours with an area of less then 2 μm^2 are considered spurious spots and non counted.

The second parameter used for the removal of spurious spots is the ratio between the contour area and the area corresponding to the rectangle enclosing the contour. Discarding contours with a ratio below 10% lets discarding contours which have a wire-like shape and therefore cannot be considered as bacteria.

Fig. 3 shows an example of the obtained results where the identified contours, both for the bare metal and for the coated one are shown in green color.

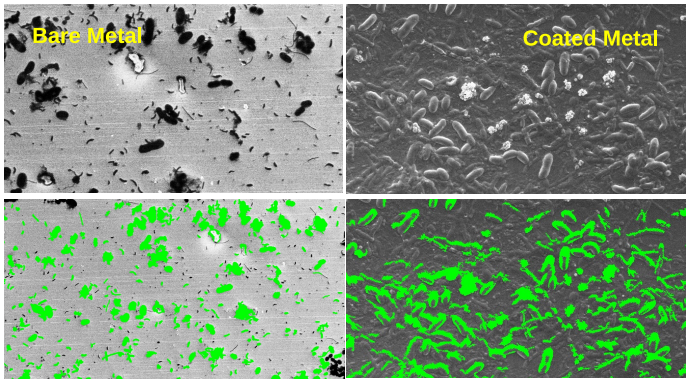

Fig. 3. Example of processed images, where bacteria are labeled in green.

IV. Use of the imaging procedure on coated samples

The described procedure has been used to assess the antimicrobial behavior of different epoxy coatings containing silver nano-particles.

Three sets of samples have been produced following the procedure described in [24]. The silver precursor (Silver hexafluoroantimonate, $AgSbF_6$) is mixed with the epoxy resin and the photoinitiator, $2,2-dimethoxy-2-phenyl\ Acetophenone$ (DMPA, 2 wt%), until an homogeneous mixture is obtained. After that, it is spread on the metal surface (steel substrates have been used) and irradiated by UV light in order to cure the epoxy resin. During the polymerization process, silver nanoparticles are produced in-situ directly in the polymeric matrix, assuring a proper distribution inside the material. Two concentrations of the silver precursor have been chosen for these tests: 1 wt% and 3 wt% (in the following, these samples will be named respectively 'Ag1' and 'Ag3'). Epoxy coatings without the addition of silver precursor have been realized as a reference material (they will be referred to as 'EP'). As reported in [25], it should be also specified that obviously the silver nanoparticles concentration is not equal to the amount of precursor added to the resin, but lower.

In order to test the antimicrobial properties of the different coatings, they have been immersed in a solution containing bacteria for 300 hours at a temperature of $30\pm3°C$; wastewater was used as inoculum for bacteria.

Fig. 4 shows an example of FESEM micrographs and of the corresponding identified bacteria of three different coatings after the immersion test. It is easy to observe how in the absence of silver nanoparticles dispersed in the polymeric matrix, bacteria attach on the surface, clustering together. The immersion of about 300 hours is not long enough to cause

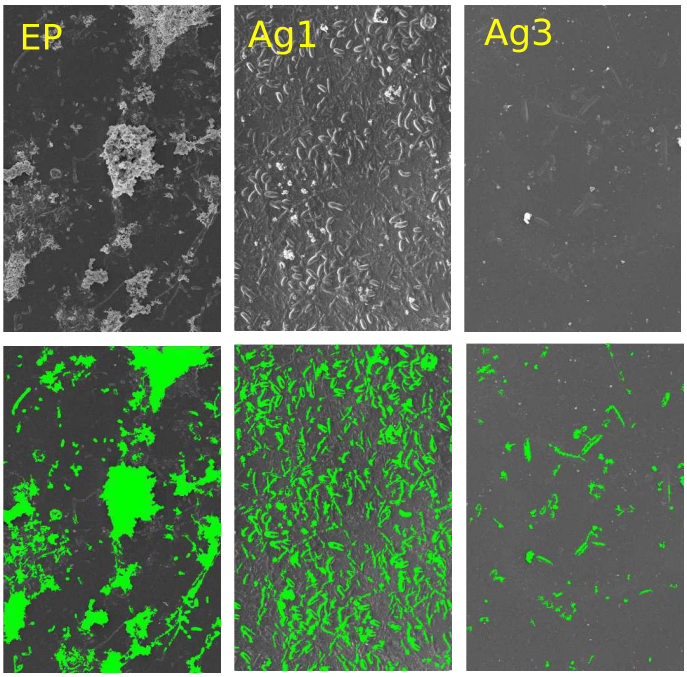

Fig. 4. Example of FESEM micrographs and identified contours on three samples after immersion in a solution containing bacteria: EP, epoxy coating without silver nanoparticles, Ag1, epoxy coating containing 1 wt% of Silver precursor, Ag3, epoxy coating containing 3 wt% of Silver precursor.

the formation of defects or damages on the epoxy coating, but fouling surely affects the protective properties of the material.

When silver nanoparticles are present in the coating, bacteria adhesion is inhibited by silver biocidal effect. A first effect can be observed already at a low concentration as 1 wt%. Bacteria are no more able to form big aggregates, even if they are still present and colonize almost uniformly the sample surface. It must also be observed that through this imaging technique the information that is acquired is related to the attachment of bacteria on the sample, without any clue if they were still living at the end of the test.

Increasing the concentration of silver precursor to 3 wt%, even better results can be observed. Actually, bacteria adhesion on the coating surface is almost completely inhibited and only few microorganisms are present on it. Moreover, even some of the identified clusters seem to be mostly due to coating roughness more than to bacteria aggregates.

V. Conclusions

Ag-doped epoxy coatings appear as an interesting solution when antimicrobial properties are required. The addition of silver precursor at a concentration of only 3 wt% already proves a good antifouling behavior, inhibiting bacteria adhesion on the coating surface.

Even if dealing with non-conducting samples, in which the image contrast is low, the algorithm was able to process the images correctly, identifying bacteria and quantifying the amount of sample surface colonized by them.

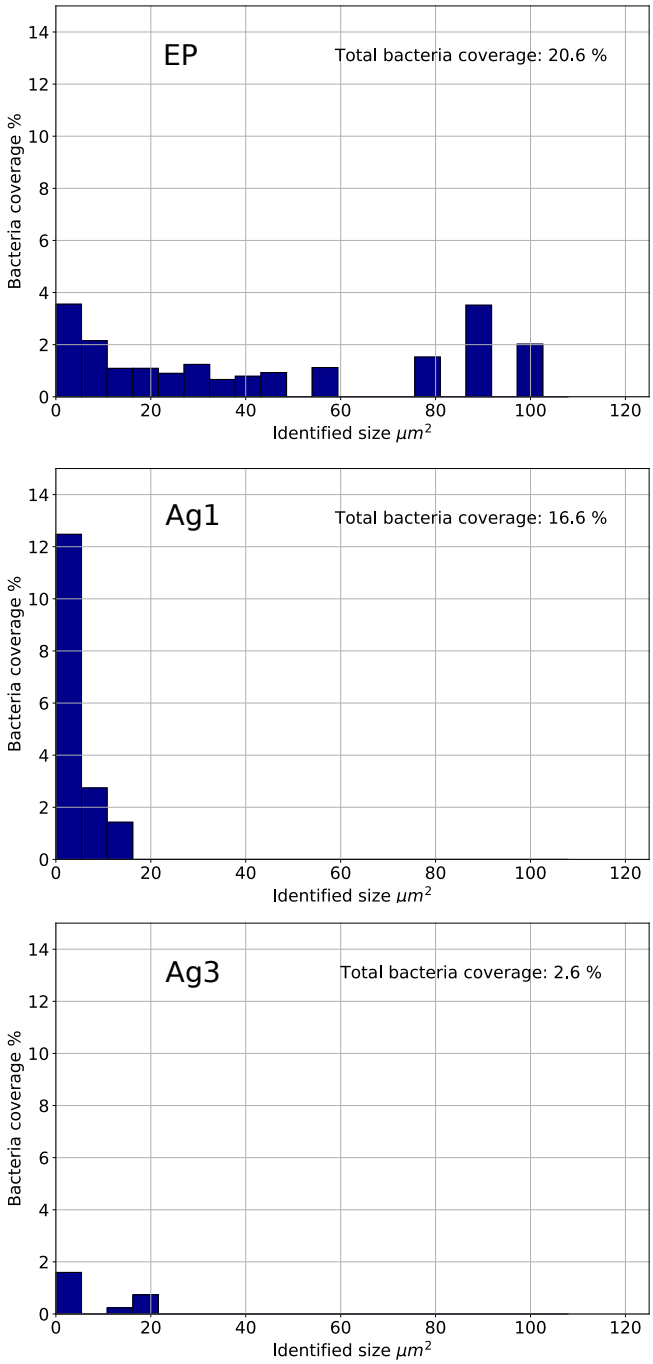

Fig. 5. Histograms obtained by estimating the bacteria attached to three different types of samples: EP, epoxy coating without silver nanoparticles, Ag1, epoxy coating containing 1 wt% of Silver precursor, Ag3, epoxy coating containing 3 wt% of Silver precursor.

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