PROOF-OF-CONCEPT EXPERIMENT REPORT

SERS SUBSTRATE FOR FAST BACTERIA DETECTION

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ISTITUTO OFFICINA DEI MATERIALI

Proof-of-Concept: introduction and general description

Main aim of the proposal:

A company specialized in the production and marketing of disinfection systems for surfaces and environments, requested our support to verify the possibility of developing a rapid and cost-effective method for the quantitative and qualitative analysis of ambient air samples.

Currently, the samples are taken with a SAS (Surface air System) sampler: the SAS carries out the sampling from the air duct directly on a Petri dish for bacterial cultures. After sampling, the plate is sent to a microbiological analysis laboratory for colony counting and recognition of the bacterial strains involved in the contamination. The bacteria adhering to the agarose gel surface, if alive, grow and produce countable colonies, to trace the initial number of bacteria present.

The timing required to obtain the results on the microbiological analysis, in the order of days, does not allow the company to have an immediate result and therefore act promptly with their treatment.

The purpose of the POC experiment is to verify if nanotech approach can provide a substrate which is effective in collecting and detecting microorganism (e.g. bacteria) in air samples collected by SAS sampler. What has been proposed and tested is the development of a SERS (Surface Enhanced Raman Spectroscopy) platform, for the qualitative and quantitative analysis of microorganisms present in the air, to be integrated with the SAS sampling tool used by the company. SERS platform is a substrate typically based on metal thin films, which is capable to amplify the Raman signal coming from the absorbed species. In this case the targeted SERS platform needs to be also effective in microorganism collection. If successful, the SERS platform, capable of detecting the presence of bacteria, could potentially offer the possibility of a quantitative and qualitative analysis of the microorganisms present in the sample by Raman spectroscopy, in a precise and rapid manner.

Three options have been tested: 1) simple metal thin film on a Petri dish; 2) metal film coated with Self-Assembled monolayer (SAM); 3) microstructured PDMS film with metal coating.

Work plan:

1st step: direct metallization of petri dish and test

2nd step: functionalisation of the petri dish by SAM coating and test

3rd step: Micro structures on the petri dish and test

Measurement author and place: The fabrication process and the characterisation of each treatment has been performed by dr. Simone dal Zilio and Martina Conti at Istituto Officina dei Materiali- CNR.

Proof-of-Concept experiment details

1st step: direct metallization of petri dish and test

The first test to verify the effectiveness in the use of the SERS technique for the detection and count of bacteria sampled by SAS was to generate an active surface based on thin Ag film. For this purpose, suitable

petri dishes were processed ad hoc so as to be covered with a thin film of SERS-active metal; in detail, the 60 mm diameter Petri dishes were subjected to an oxygen plasma process (40W, 100 V, 30 s) to improve their adhesion as a function of the subsequent deposition step, by e-gun evaporation, of a thin film composed of titanium (20 nm) and Ag (100 nm).

Figure 1 Example of plates metallized with Ti-Ag

The plates sent to the company were subjected to the same sampling with SAS together with others with agarose as a reference. Below is an example of the test reports sent to us by the company, on the results of the microbiological analyzes carried out on the reference Petri dishes, before and after the sanitization process.

From the analysis in optical microscopy, an insufficient detection of microorganisms was found (see image below), probably due to the poor interaction with bacteria of the metallized surface. Given the absence of bacteria on the surface, the samples produced were not measured by Raman spectroscopy.

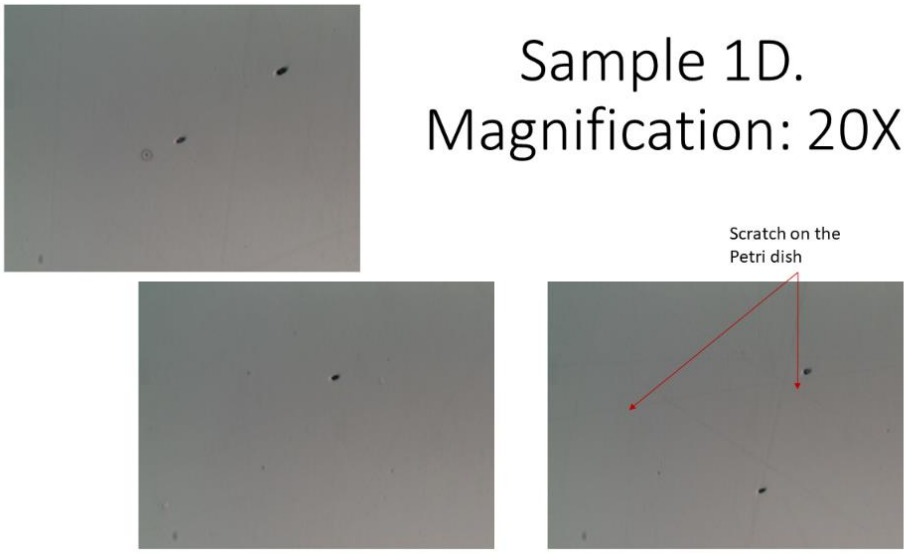

Figure 3 Examples of images acquired with an optical microscope. The particles present seem to be attributable to dirt or contamination not of bacterial origin.

2nd step: functionalisation by APTES of glass-based substrates

With the aim of improving the adhesion of bacteria, the effect of an ad hoc functionalization was explored that could allow a better affinity with the microorganisms sampled through the SAS. It is known, in fact, that the self-assembled monolayers (SAMs) of APTES (aminopropyl tri-ethoxy silane), thanks to the presence of amino functional groups, are able to favor the adhesion of microorganisms.
Since the material constituting the Petri dishes (PEG), that is PS, cannot be functionalized in this way, nor can it undergo the required thermal processes (up to 120 ° C), it was decided to produce new SERS substrates in convenient material, to be inserted later inside the Petri dishes.
For this purpose, body slides (diameter 24 mm) were used, appropriately metallized in the same way as the petri dishes referred to in point 1. The slides thus prepared were then functionalized with a process developed in our laboratories from the vapor phase (CVD).
The silver film can in fact be functionalized if subjected to an appropriate treatment in oxygen plasma. In the same way, control samples of simple glass (not metallized) were prepared for which the efficacy of the functionalization process with silanes is well known.

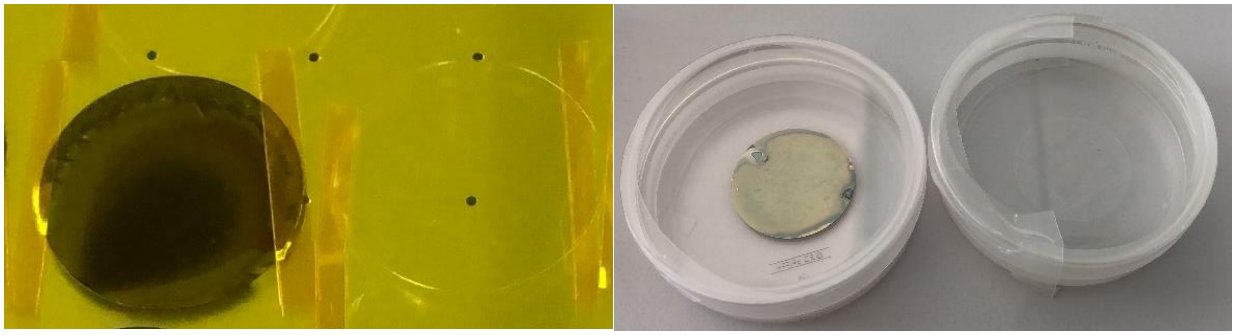

Figure 4 Slides, metallized or not, after functionalization and insertion into petri dishes

The substrates thus created were glued with double-sided adhesive tape to the bottom of the Petri dishes.

The samples were then sent to the company, which proceeded to carry out the same air sampling (with SAS) on our substrates and on the Petri dishes with standard agar.

From the optical microscopy analyzes, carried out in our laboratories, unlike the control on standard plates, it was not possible to detect the presence of bacteria except in very small numbers and without reproducibility or consistency with respect to the controls (Fig 5).

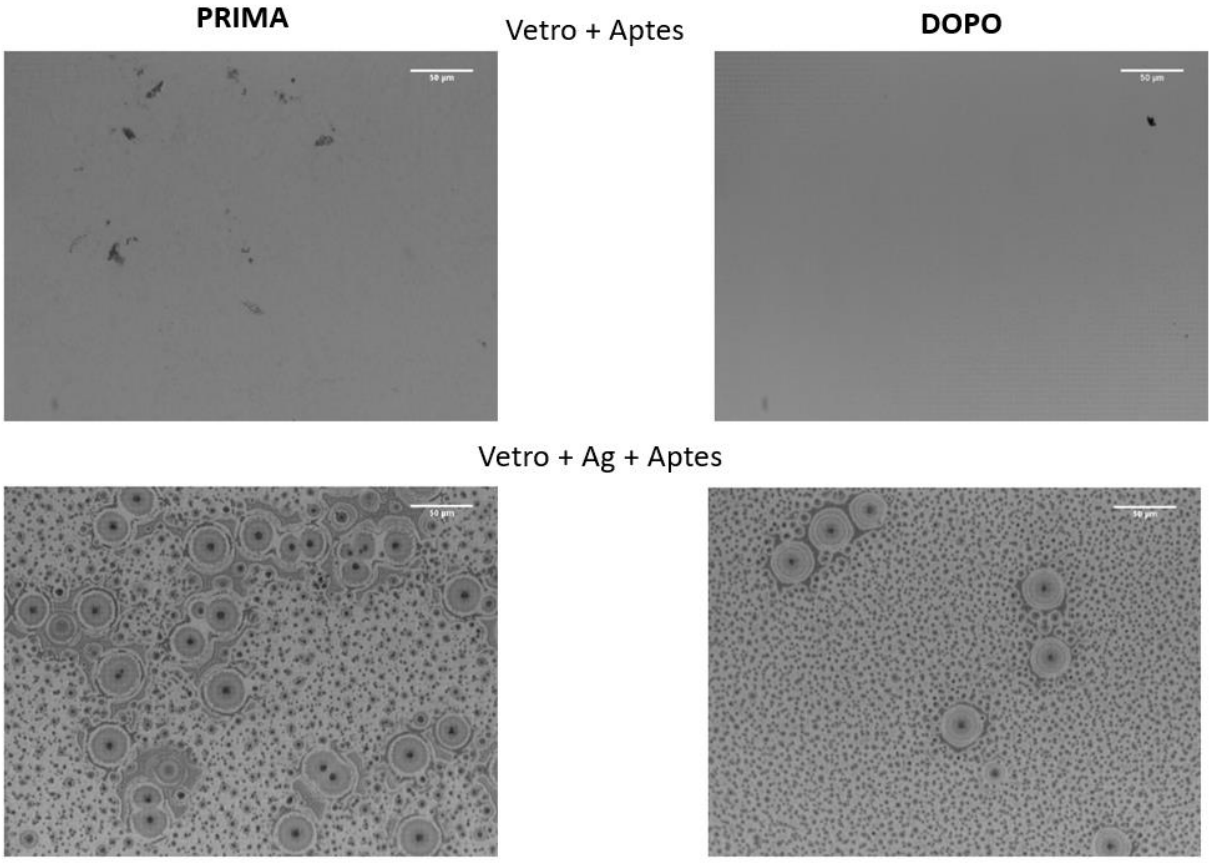

Figure 5 Optical microscopy images of the glass samples, with or without Ag metallization, functionalized with APTES, BEFORE and AFTER the sanitization process.

We hypothesize that functionalization with APTES is no longer effective when the sampling is carried out dry.

3rd step: microstructures of PDMS

Given the inefficient adhesion of microorganisms on glass substrates, new substrates made of microstructured PDMS (Polydimethylsiloxane) were tested.
The substrates of microstructured PDMS consist of a silicone sheet on the surface of which are made of arrays of micropillars with dimensions: 2.5 x 6.8 µm (diameter x height) with a period of 4 µm. The structures were obtained by "replica molding" of a silicon mold produced by UV lithography and dry etching processes, starting from liquid precursor and subsequent polymerization.
Substrates were prepared with a base and catalyst ratio of "10: 1".
The samples obtained, after an O2 plasma treatment (40W, 100 V, 30 s), were subjected to thermal evaporation to deposit first a titanium adhesion film (20 nm) and then an Au film (45 nm) .
Once ready, the PDMS substrates were fixed with clear double-sided tape to the surface of 92 mm Petri dishes.
Similarly, control samples of non-metallized microstructured PDMS were prepared to highlight any contaminants deriving from the process of thermal evaporation of the thin film of Au.

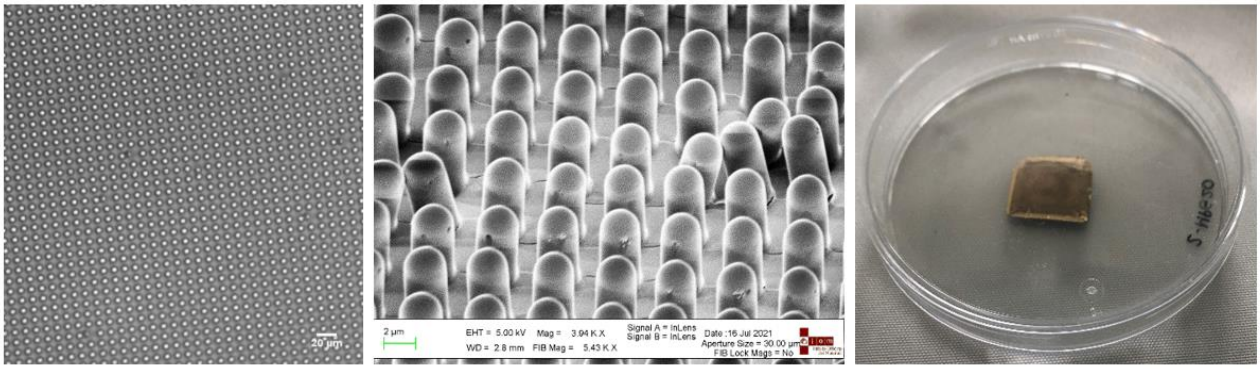

Figure 6 Image of the micro pillars acquired by optical and SEM microscopy, and the substrate of PDMS glued on a 92 mm Petri dish.

Below, an image (acquired with an optical microscope and SEM) of the substrates in microstructured PDMS coated with Au.
The samples were then sent to the company, which proceeded to carry out the same air sampling (with SAS) on our substrates and on the Petri dishes with standard agar.
From the first images, acquired under an optical microscope, structures compatible with cellular organisms were evident only in the samples metallized with gold. It was thus decided to carry out an electron microscopy analysis, which demonstrated how the displayed formations were micropillars disjointed from the initial array (Fig. 7).

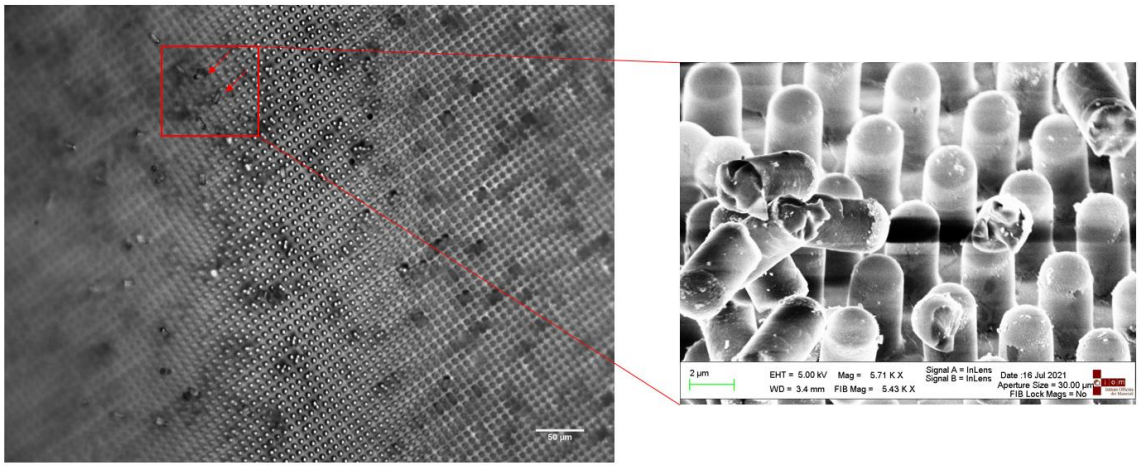

Figure 7 On the left, the optical microscopy image of the Au-coated micropillars on which structures morphologically similar to the bacterial ones were found. On the right, the SEM image that highlights how these structures were disjoint pillars.

In any case, from an in-depth analysis of the images it was possible to identify additional structures of a size compatible with the bacterial ones. It was thus decided to proceed with a treatment of the substrates with Giemsa type staining, as it is capable of marking only cellular structures.

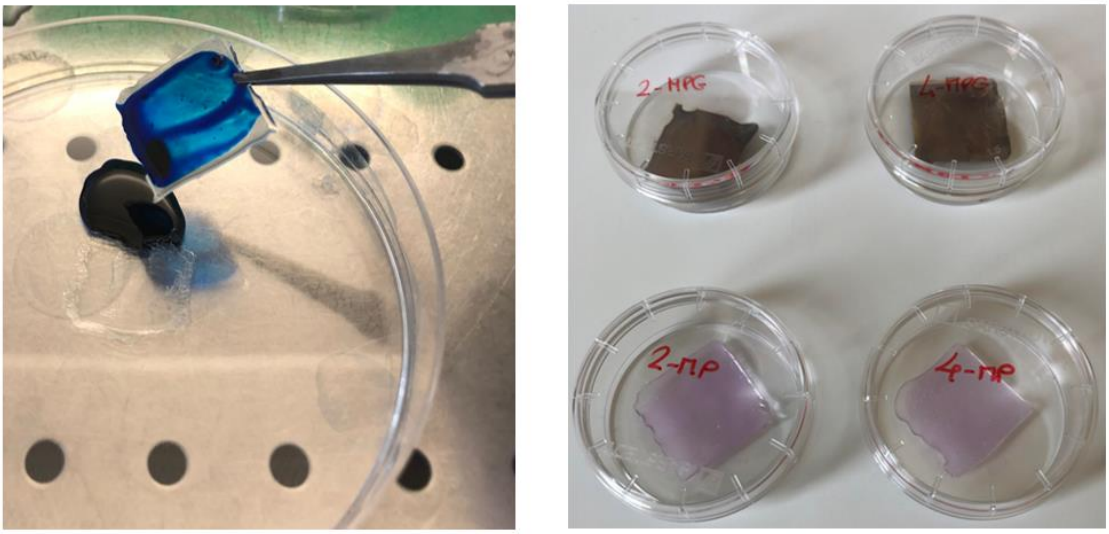

Figure 8. Left, a specimen subjected to Giemsa staining. On the right, the metallized (top) and non-metallized (bottom) samples after Giemsa staining.

From the results, once again, only the metallized samples appear to have marked formations. In addition, the non-metallized samples, after coloring, took on a conformation that made microscopic investigation difficult. From the analyzes, it was not possible to clarify with certainty whether the formations found on the metallized samples were actually bacteria or residues from the gold evaporation processes on the substrates. In addition, the samples taken before and after the sanitization treatment, as well as presenting insufficient adhesion of microorganisms, did not seem to differ from each other.
However, the samples taken on the agar plates and analyzed by the company were positive for the presence of bacteria, albeit to a small extent. Below is the test report of the microbiological analyzes sent to us by the company.

Given the inefficiency of adhesion of bacteria to the surface of the substrates of microstructured PDMS, it has been hypothesized that the O2 plasma treatment to which the substrates are subjected before the evaporation process could be one of the causes; Treatments in plasma of PDMS are known to cause a sort of vitrification of the surface, as the organic part of the material is eliminated. The micropilllars treated in this way can lose some of that elastic properties that increase the "capture" capacity of the bacteria themselves. For this reason, it was decided to prepare two sets of samples “15: 1” and “20: 1” which differ in the ratio of base and cross-linking agent used during the preparation of the PDMS solution. In particular, this ratio influences the rigidity of the substrates obtained. In fact, with the increase of the base concentration there is a decreasing rigidity of the micropillars.
After the preparation of the two solutions, using values that differ in the ratio between base and cross-linking agent, the substrates were subjected, like the previous ones, to treatment in plasma O2 (40W, 100 V, 30 s) and then to evaporation for the deposition of the Au film.
Once ready, the substrates were glued with clear double-sided tape to the surface of 92mm Petri dishes.
From the analyzes carried out with optical microscopy it was not possible to detect the presence of microorganisms neither in the PDMS structures with a 15: 1 ratio, nor in those with a 20: 1 ratio. Below are the images of a 15: 1 sample under optical microscopy of the substrates, before

and after the sanitization process.

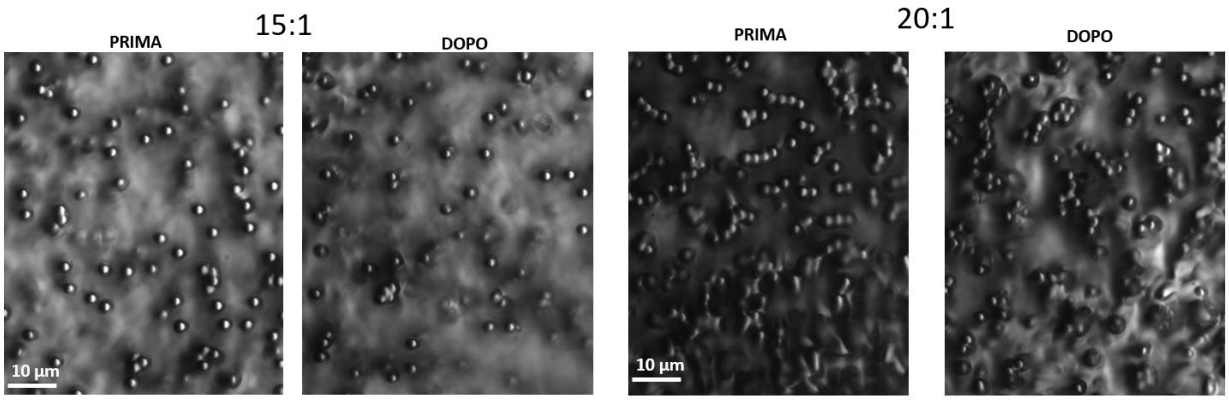

Figure 10. Images acquired under an optical microscope of the substrates with a ratio of 15: 1 (left) and 20: 1 (right) on which the company sampled, before and after the sanitization process.

Number of samples	Date	Sample description	Result
5	31/05/2021	Petri dish 60 mm covered by Ti/Ag	No bact. adhes.
5	8/06/2021	Petri dish 60 mm covered by Ti/Ag	No bact. adhes.
5	18/06/2021	Set1: Petri dish 60 mm + Glass+APTES (G+Ap)	No bact. adhes.
5	18/06/2021	Set2: Petri dish 60 mm + Glass+Ag+APTES (G+Ag+Ap)	No bact. adhes.
4	08/07/2021	Set1: Micropillars PDMS	No bact. adhes.
4	08/07/2021	Set2: Micropillars PDMS + Gold	No bact. adhes.
5	10/09/2021	Set1: Micropillars PDMS (MPG15)	No bact. adhes.
5	10/09/2021	Set2: Micropillars PDMS (MPG20)	No bact. adhes.

Summary of results

Three different SERS substrates have been tested, with the purpose of verifying their suitability to adhesion of bacteria and SERS analysis. None of them resulted to be effective in the collection of bacteria, at least when combined with the SAS collection procedure used by the company.

Advices

The tested approach does not seem to be usable in the proposed solution: the main reasons for failure are to be found in the poor affinity of the bacteria with surfaces similar to those used. It is suggested to find an alternative route, focusing the design first on improving bacterial acquisition on devices.

This report has been written by Simone Dal Zilio, 04/11/2021