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Object: Research contract commissioned to carry out the following activity: "to evaluate the anti-SARS-CoV-2 efficacy of a functionalized surface".

The research activity reported in the object was focused on the evaluation of the anti-SARS-CoV-2 activity of a functionalized surface by means of a suitable cellular model.

Specifically, the assays that were set up included the use of known amounts of virus and, adopting Vero E6 cell cultures, the analysis of the reduction of viral infectivity following contact with the treated materials with anodizing with silver ions "GHA".

Materials and Methods

Cell culture

The African green monkey kidney Vero E6 (ATCC CRL-1586) cell line was purchased from the American Type Culture Collection (ATCC), and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2. Vero E6 cells are highly sensitive and permissive to SARS-CoV-2 replication.

Viral stock preparation and titration

The SARS-CoV-2 2019-nCoV/Italy-INMI1* clinical isolate was adopted. Viral stock was obtained by infecting cellular monolayers of Vero E6 with a low multiplicity of infection (m.o.i., equal to 0.01 PFU/cell, where PFU stands for Plaque-Forming Units). In the presence of a cytopathic effect affecting more than 80% of cultures, both monolayer cells and supernatans were collected and subjected to three cycles of freezing-thawing and subsequent centrifugation at low speed to eliminate cellular debris. The supernatant was then aliquoted and kept frozen at -80°C until use.

Titer of viral suspensions was determined by plaque assay. To this end, Vero E6 cells were seeded at a density of 10^5 cells/ml and infected in the presence of scalar dilutions of the sample under evaluation for 1 hour at 37°C. Next, cells were maintained at 37°C in DMEM at 2% (v/v) of FBS plus 0.75% (v/v) of carboxymethylcellulose. Forty-eight-72 hours later, cells were fixed in formaldehyde and stained with crystal-violet. Plaques formed on the monolayer due to the cytopathic effect of the virus were then counted at the optical microscope. Viral titre was calculated by adopting the Spearman-Karber method and expressed as PFU/ml.

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To assess the reduction of viral infectivity, the procedure described in the **ISO 21702_2019 "Measurement of antiviral activity on plastics and other non-porous surfaces"** was used. Specifically, 400 µl of viral suspension with a titer of 10^7 PFU/ml were deposited on samples of the test materials measuring 4 cm x 4 cm. The inoculum was covered with a 3 cm x 3 cm film, in order to create a thin layer in contact with the material. Each sample was put in a closed petri dish to minimize evaporation. After an appropriate incubation time, the residual virus was collected using 10 ml of serum-free DMEM and titrated, in duplicate, on Vero E6 cells as specified above.

All the experiments were conducted in a Biosafety Level 3 laboratory (BSL3).

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Results

Two experiments were performed, by exposing test and control samples to SARS-CoV-2 for 30 minutes, 1 hour and 2 hours. For each experiment and for each time point, 3 samples of treated material and 3 of control material were used. The viral titer was evaluated by plaque assay. The results obtained are illustrated in the following Tables, each representative of each individual experiment.

Specifically, viral titer is calculated by adopting the formula reported at the section 8.1 of the adopted ISO, i.e.:

$N = (10 \times C \times D \times V) / A$

Where

N is the infectivity titer of virus recovered per cm^2 of test specimen;
C is the average number of plaque counted for the duplicate wells;
D is the dilution factor for the wells counted;
V is the volume of the medium added to the specimen (10 ml);
A is the surface area of the cover film, in cm^2 (9 cm^2)

In both experiments, the conditions set out in sections 8.2.2/8.2.4 of the reference ISO were valid.

In particular, the viral titer recovered in the case of control samples was always higher than 6.2 x 10^2 PFU/cm^2.

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Furthermore, the titer obtained from the samples recovered immediately after contact with the control material (N_0) was always between 2.5×10^5 PFU/cm^2 and 1.2×10^6 PFU/cm^2 as shown in each Table.

The data reported in the Tables are expressed as antiviral activity "R", calculated as reported in the section 8.3 of the reference ISO, namely:

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t$$

Where

R is the antiviral activity;
*U*0 is the average of the common logarithm of the number of plaques recovered from the three untreated test specimens immediately after inoculation, in PFU/cm^2;
*U*t is the average of the common logarithm of the number of plaques recovered from the three untreated test specimens at the time "t" post-inoculation, in PFU/cm^2;
*A*t is the average of the common logarithm of the number of plaques recovered from the three treated test specimens at the time "t" post-inoculation, in PFU/cm^2.

Finally, the % of reduction in viral titer was calculated by comparing the values obtained for the samples recovered from the test material and those resulting from the samples incubated with the control material at the same time post-inoculation.

Table experiment #1

	30 minutes		1 hour		2 hours	
	titer	**% reduction**	**titer**	**% reduction**	**titer**	**% reduction**
CONTROL	1.9*10^5 PFU/cm^2		1.1×10^5 PFU/cm^2		1.4*10^5 PFU/cm^2	
TEST	5.1*10^4 PFU/cm^2	**73%**	2.6*10^4 PFU/cm^2	**76%**	1.1*10^4 PFU/cm^2	**92%**
R	**0.57**		**0.62**		**1.1**	

N_0 = 3.5*10^5 PFU/cm^2

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Table experiment #2

	30 minutes		1 hour		2 hour	
	titer	**% reduction**	**titer**	**% reduction**	**titer**	**% reduction**
CONTROL	1.5*10^5 PFU/cm^2		1.4*10^5 PFU/cm^2		1.2*10^5 PFU/cm^2	
TEST	4*10^4 PFU/cm^2	**73%**	2*10^4 PFU/cm^2	**86%**	9*10^3 PFU/cm^2	**93%**
R	**0.57**		**0.85**		**1.12**	

N_0= 3.97*10^5 PFU/cm^2

In conclusion, taking into account the natural decay of the viral infectivity outside the intracellular environment, we can conclude that the treatment of the material under evaluation has the ability to decrease the SARS-CoV-2 titer by more than 1 logarithm, after 2 hours of contact.

Padua, 20th September, 2021

Signature

Prof. Maria Cristina Parolin

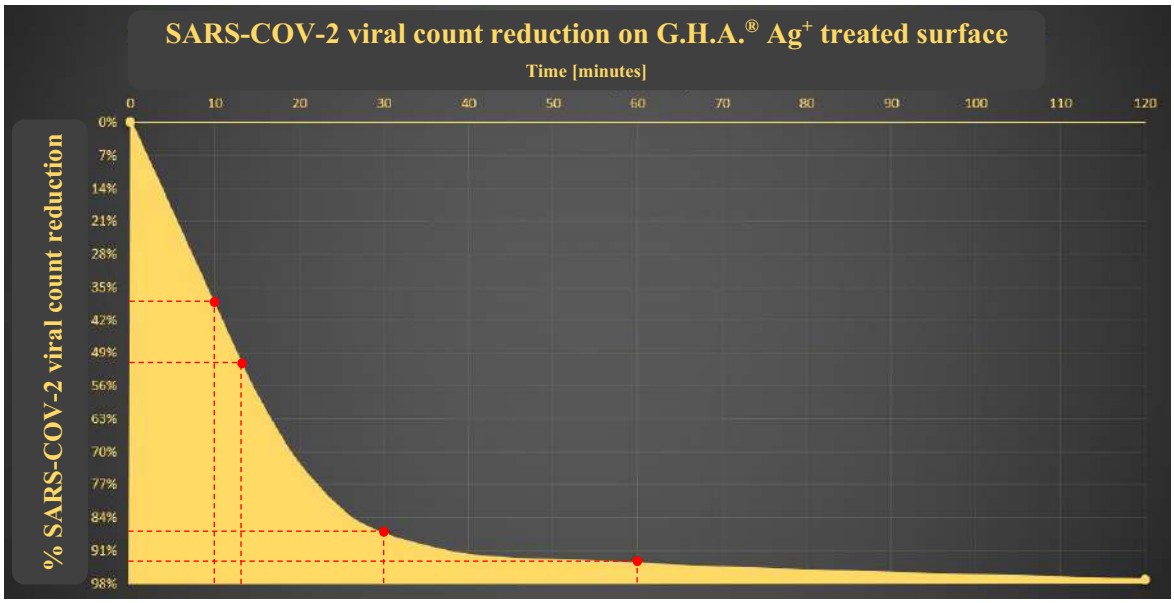
ANTIVIRAL EFFECT OF SURFACES ON SARS-COV-2

Material	G.H.A.®	Copper	Carton	Stainless steel	Plastic
Half-life	< 15 min	1 h	3 h	6 h	7 h
No viability	2 h	4 h	24 h	48 h	72 h

Data extracted from the articles "Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1 (Doremalen et al. 2020)", "Sustainability of Coronavirus on Different Surfaces (Suman et al. 2021)" and from the relation of Dipartimento di Medicina Molecolare of Università degli studi di Padova (Parolin, 2021-8-18).

As can be seen in the table, copper surfaces, that up until today were believed to be the most inhospitable for the SARS-COV-2 virus, allow the survival of the virus for up to 4 hours.

The graph below was obtained starting from the data included in the relation of Dipartimento di Medicina Molecolare of Università degli studi di Padova (Parolin, 2021-8-18). It is evident that the viral count of SARS-COV-2 on G.H.A. treated surfaces is more than halved after 15 minutes, after one hour it is reduced by 94% from the original value and it is reduced by 98% after two hours.



The value in the ordinate represents the reduction percentage of the viral count on the G.H.A. treated sample. It is expressed as a percentage related to the original viral count.

Article links:

Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1 (Doremalen et al. 2020) - https://www.nejm.org/doi/10.1056/NEJMc2004973

Sustainability of Coronavirus on Different Surfaces (Suman et al. 2021) - https://www.jcehepatology.com/article/S0973-6883(20)30062-1/fulltext