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Customer Name	Maeda-Kougyou Japan 218-2 Norimatsu Yahatanishi ward Kitakyushu City Japan
Contact	Dr. Khaled Hussein
Customer PO no.	N/A
Test Requested	BS ISO 27447: 2009 Fine ceramics (advanced ceramics, advanced technical ceramics) – modified for virucidal assessment against Influenza A
Sample Description	Stainless steel: non-coated and coated with Miracle Titanium (Primary and MVX)
Date of Receipt	8th August, 2012
Project Number	ASCR092018
Report Date	5th October, 2012

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Description of Test Items

The following items were tested. The samples to be tested all measured 50 x 50 mm.

Test Item	Product Code
Treated Stainless Steel	ASC002198
Untreated Stainless Steel	ASC002199

Introduction

The purpose of the project was to ascertain the effect of the MVX coating, when applied to stainless steel following exposure to UV and incandescent light, on Influenza A virus viability as per the client's request. It was agreed that the test would be performed in accordance with "BS ISO 27447: 2009 Fine ceramics (advanced ceramics, advanced technical ceramics). Test method for antibacterial activity of semiconducting photocatalytic materials". However some alterations were made to the test method to allow it to be used for assessing virucidal activity. The test was performed in triplicate.

Influenza A was selected because:

- it can survive for several hours in the environment and is resistant to various parameters such as pH, temperature and humidity changes
- it is transmitted via indirect contact with contaminated objects and surfaces or via direct contact with other people and/or via airborne transmission
- it is associated with Healthcare-Acquired Infections (HCAI)
- it can be grown and quantified in cultured cells for determination of virucidal effects

Procedure

<u>Virus Stock Preparation</u>

1. A stock of Influenza A ATCC VR-1469 was aliquoted and stored at –80°C until testing. On the day of the test set-up, an estimate of the virus titre was determined by standard 50% Tissue Culture Infectious Dose ($TCID_{50}$) in the Madin-Darby canine kidney (MDCK) cell line (Vircell, Serosep) with 10-fold serial dilutions of samples. MDCK cells were grown in Eagle's Minimal Essential Medium (EMEM) with 10% fetal bovine serum, trypsin and antibiotic-antimycotic.

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Photocatalytic Reaction Set-up: Stainless steel (Film adhesion method)

1. The stainless steel, film and glass slides were autoclaved on the day prior to test set-up. The treated and untreated stainless steel samples were individually placed in labelled sterile petri-dishes on top of a glass slide that was resting on sterile filter paper moistened with phosphate buffered saline (PBS).
2. The light intensity of the UV and incandescent light reaching the surface of the centre of the petri plates was adjusted to 0.13 mW/cm^2. The measurement was performed using an ILT1700 radiometer/photometer and probe (International Light Technologies, Peabody, MA, USA).
3. A 100 µl aliquot of Influenza A virus suspension was placed on top of the treated and untreated stainless steel samples and immediately covered with sterilised film. The film was carefully pushed down so that the liquid spread evenly underneath it. The lids of the petri dishes were placed on top.
4. Treated and untreated samples were kept in a dark place or exposed to the light source (UV and incandescent light) for 6 hours.
5. Three untreated samples were immediately withdrawn at t = 0 (time zero) to ascertain the recovery of the virus under test immediately after inoculation.
6. The titer of the thawed virus stock was determined at t = 0 by $TCID_{50}$.
7. Influenza A was extracted from the stainless steel tiles with 10ml of culture medium containing 0.125% bovine serum albumin and 1 µg/ml trypsin.

2. A 1 ml aliquot of the extracted virus samples were 10-fold serially diluted in cell culture medium and 100 µl/well was added to quadruplicate wells of a 96-well plate containing 80% confluent MDCK cells. The plates were incubated at 35°C, 5% CO_2 until development of viral cytopathic effects were observed. Titers of Influenza A were determined in each sample using $TCID_{50}$.
3. The same procedure was followed for the untreated and treated samples that had been incubated in the dark (controls) for 6 hours or irradiated with UV and incandescent light for 6 hours. The plates were incubated at 35°C, 5% CO_2 until development of virus cytopathic effects were observed. Titers of Influenza A were determined in each sample using $TCID_{50}$ assay.
4. Following incubation, wells with evidence of virus cytopathic effects (CPE) were counted and converted to $TCID_{50}$ units/ml ($TCID_{50}$/ml) using the Karber Formula (formula 1 below). The $TCID_{50}$/ml results were converted to plaque forming units/ml (PFU/ml) as shown in

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formula 2 below. Following conversion the photocatalyst antiviral activity values were calculated using formulas 3 and 4.

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FORMULAs

1) *Karber Formula for calculating $TCID_{50}$*

The quantity of virus in a specified suspension volume (e.g. 0.1 ml) that will infect 50% of a number (n) of cell culture microplate wells, or tubes, is termed the Tissue Culture Infectious Dose 50 [TCID50].

$\log TCID_{50} = L - d (S - 0.5)$, where:

L = log of lowest dilution used in the test

d = difference between log dilution steps

S = sum of proportion of "positive" tests (i.e. cultures showing CPE)

2) *Conversion of $TCID_{50}$/ml into plaque forming units/ml (pfu/ml)*

$TCID_{50}$/ml of each sample x 0.69 = pfu/ml x dilution factor (100)

The PFU of a solution is the concentration of virus particles in a solution that are capable of lysing host cells and forming a plaque (zone of destruction) in a cell culture. For example, if a solution has a PFU of 1000 pfu/ml, then 1 ml of that solution contains enough virus particles to form 1000 plaques.

Photocatalyst antiviral activity value calculations

3) $R_L = [\log(B_L / A) - \log(C_L/A)] = \log[B_L/C_L]$

R_L is the photocatalyst antiviral activity value after light exposure

A is the average titer of viable virus of non-treated samples just after inoculation

B_L is the average titer of viable virus of non-treated specimens after light exposure

C_L is the average titer of viable virus of photocatalytic treated specimens after light exposure

4) $\Delta R = \log[B_L/C_L] - \log[B_D/C_D]$

ΔR is the photocatalyst antiviral activity value with UV irradiation

B_D is the average titer of viable virus of non – treated specimens after being kept in a dark place

C_D is the average titer of viable virus of photocatalytic treated specimens after being kept in a dark place

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Results

Influenza A

Sample description	Virus Titer	Average Virus Titer	Log values
U.T 1 t = 0	3.88E+08	7.69E+08	8.886
U.T 2 t = 0	1.23E+09		
U.T 3 t = 0	6.90E+08		
U.T Dark 1 t = 6 hrs	6.90E+07	8.70E+07	7.940
U.T Dark 2 t = 6 hrs	6.90E+07		
U.T Dark 3 t = 6 hrs	1.23E+08		
TR Dark 1 t = 6 hrs	3.88E+07	6.69E+07	7.825
TR Dark 2 t = 6 hrs	3.88E+07		
TR Dark 3 t = 6 hrs	1.23E+08		
U.T U.V 1 t = 6 hrs	1.23E+07	8.70E+06	6.940
U.T U.V 2 t = 6 hrs	6.90E+06		
U.T U.V 3 t = 6 hrs	6.90E+06		
TR U.V 1 t = 6 hrs	2.18E+05	1.54E+05	5.188
TR U.V 2 t = 6 hrs	1.22E+05		
TR U.V 3 t = 6 hrs	1.22E+05		

Note
Dark = Incubated in dark conditions at room temperature
U.T = Untreated samples
U.V = UV light exposed for 6 hours
TR = Treated

Photocatalyst antiviral activity value calculation

R_L = log[8.7 x 10^6/1.54 x 10^5]=
= log[56.49]
= **1.752** *Photocatalyst antiviral activity value after light exposure*

ΔR = log[8.7 x 10^6/1.54 x 10^5] – log[8.7 x 10^7/6.69 x 10^7]
= log[56.49] – log[1.3]
= 1.752 – 0.114
= **1.638** *Photocatalyst antiviral activity value for UV irradiation*

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Discussion and Conclusion

In this test, the ISO 27447:2009 protocol was modified to enable a MVX-coated stainless steel surface to be tested with viruses.

In order to understand the data it must be noted that R_L values account for the reduction of viability caused by the exposure of the treated surfaces to UV irradiation and incandescent light for 6 hours. It does not take into account any reduction of viability that occurred in the dark over the 6 hour incubation period. In contrast, ΔR values address the reduction of viability caused by the coated steel becoming light activated while accounting for the reduction in viability caused by the same coating in the dark environment.

From a log recovery at t = 0 of 8.886, the plaque forming unit (PFU) recovery was reduced to 7.940 and 7.825 from untreated and treated samples incubated in the dark respectively. Untreated and treated samples incubated in the presence of UV light demonstrated log recoveries of 6.94 and 5.188 respectively.

There are very few studies carried out to date that have examined the effect of photocatalytic disinfection using TiO_2 on influenza virus viability. It is clear from this study that the MVX coating has a significant photocatalytic virucidal effect against Influenza A, with a photocatalyst antiviral activity value after UV irradiation of 1.638. Nakano et al. (1) also found that influenza A was inactivated using this disinfection method, although they used different methods and formulae to calculate their results.

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Reference

1) R. Nakano, H. Ishiguro et al. Photocatalytic inactivation of influenza virus by titanium dioxide thin film. Photochem. Photobiol. Sci. (2012), 11, 1293-1298.

*** End of Report***

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