Antimicrobial activity of metal oxide microspheres: an innovative process for homogeneous incorporation into materials

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Abstract

Aims: To investigate the potent control of microbial surface contamination of an innovative process which consists in incorporating metal oxide microspheres homogeneously into materials.

Methods and Results: Spherical microspheres containing zinc and magnesium oxides synthesized via a one-step manufacturing process (Pyrolyse Pulvérisée®) and incorporated into different plastic matrices were evaluated for their antimicrobial activity according to JIS Z 2801 standard. A significant activity was observed for microsphere-added polyethylene coupons with a reduction of all tested bacteria populations, including Gram negative and Gram positive even expressing acquired antibiotic resistance (*Escherichia coli* ESBL, *Staphylococcus aureus* metiR). An antiviral activity higher than 2 log of reduction was also observed on H1N1 and HSV-1 viruses. This antimicrobial effect was dose-dependent and time-dependent for both polyethylene and polypropylene matrices. Antimicrobial activity was maintained after exposition to disinfectants and totally preserved 50 months after the preparation of the coupons.

Conclusions: Incorporated into plastic matrices, metal oxide microspheres showed significant antibacterial and antiviral activities.

Significance and Impact of Study: This is, to our knowledge, the first report on an original process incorporating metal oxide microspheres, which have specific physico-chemical and antimicrobial properties, into materials that could be used for surface contamination prevention.

Introduction

The emergence and selection of antibiotic-resistant bacteria is considered as an ever increasing Public Health problem. This threat is of particular concern when compared with the very limited number of new antimicrobial agents in the pipeline of the pharmaceutical industry and the ability of micro-organisms to be less sensitive under biofilm (Campanac *et al.* 2002; Furiga *et al.* 2015; Sabtu *et al.* 2015; Watkins and Bonomo 2016). With this in mind, new approaches are under scrutiny, for example, environmental contamination prevention and more particularly antimicrobial surfaces.

Much work on this topic has been described in the literature, using various antimicrobial compounds with the objective of obtaining growth inhibition, killing and also antiadherent and/or antibiofilm activities. Numerous assays concerning the adsorption, coating or covalent link of antibiotics to surfaces have been performed (Oprea *et al.* 2016), but this way is now considered a misuse

given the increased risk of antibiotic resistance emergence as indicated in the French national alert plan on antibiotics (Ministère de l'Emploi du Travail et de la Santé 2011) as well as the United States national antimicrobial resistance monitoring system (NARMS) integrated report proposed for combating antibiotic-resistant bacteria (FDA CDC and USDA 2015). Among other antimicrobial molecules, metallic cations and metal oxides are frequently suggested like copper or alloys (Harrasser *et al.* 2015), silver (Sondi and Salopek-Sondi 2004), magnesium oxide (He *et al.* 2016), zinc oxide under coatings, grafted or integrated in nanoparticles (Jin *et al.* 2009; de Rancourt *et al.* 2013; Pasquet *et al.* 2014; Elkady *et al.*; Pasquet *et al.* 2015; Oprea *et al.* 2016; Salarbashi *et al.* 2016). The aim of using such molecules, and in particular metal oxides, is a large antimicrobial spectrum of activity (Jin *et al.* 2009; Pasquet *et al.* 2014, 2015), but also the frequent proof of no or poor toxic effect for humans, and sometimes improvement of wound healing and/or epithelialization (Petrochenko *et al.* 2013; Pati *et al.* 2014).

The literature presents two alternative solutions, the release of the active molecule (Harrasser *et al.* 2015; Oprea *et al.* 2016) and the nonrelease with an antimicrobial effect by direct contact. Considering the ability of microbes to adhere, survive and grow on surfaces, and because the goal is to reduce biocide use and release, while limiting human and environmental contact, the second solution looks promising. One answer, called PYCLEAR™ PROTECTION, consists of integrating metal oxides (zinc and magnesium), previously described as antimicrobial (Jin *et al.* 2009; Elkady *et al.* 2015; He *et al.* 2016), under innovative green ceramic microspheres into various materials. Microspheres were incorporated into materials without any change to the existing manufacturing process, and showed significant antimicrobial activity when integrated. This process was innovative because it enhanced the contact probability between active agents and micro-organisms, so enhanced antimicrobial surface activity while not using nanoparticles considering their doubtful impact on environment and humans (Veerapandian and Yun 2011). In many studies, the antimicrobial activity of known antimicrobial surfaces, such as silver or copper alloys, was checked using the Japanese Industrial Standard (JIS) Z 2801 (2010). This standard allows the evaluation of antimicrobial properties of hard surfaces in no-growth or limited growth conditions and by improving the surface contact with micro-organisms.

The aim of the present study was to (i) show the antimicrobial properties of PYCLEAR™ PROTECTION against the referenced strains indicated in the JIS Z 2801 (2010) standard, (ii) explore the antimicrobial spectrum of activity, including some ESKAPE pathogens (MRSA, ESBL *Escherichia coli*) (Rice 2008), but also yeasts and viruses, (iii) characterize the antimicrobial surface activity by varying the microsphere concentration or the matrix, (iv) prove the activity preservation after storage or surface cleaning/disinfection, and finally to propose to end-consumers more eco-friendly efficient products that are cleaner, safer and respond favourably to the increasing regulatory, environmental and health requirements.

Materials and methods

Bacterial and yeast strains and growth conditions

Referenced bacteria were obtained from the Institute Pasteur Collection (Paris, France). To obtain the antimicrobial spectrum, the required JIS Z 2801 (2010) micro-organisms *Staphylococcus aureus* CIP 53·156 and *Escherichia coli* CIP 53·126 were tested along with others Gram positive: *Staphylococcus epidermidis* CIP 68·21, *Streptococcus pyogenes* CIP 56·41T, *Listeria monocytogenes* CIP 82·110T and Gram negative: *Pseudomonas aeruginosa* CIP 82·118, *Salmonella enterica* CIP 60·62T, *Haemophilus influenzae* CIP 102514T and *Branhamella catarrhalis* CIP 73·21T. Two bacterial strains expressing acquired mechanisms of resistance, Methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRSA) obtained from the American Type Culture Collection and a clinical isolate of Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli* were also tested. Assays were also performed on *Candida albicans* DSM 1386 obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Strains were preserved as indicated in the NF EN 12353 (2013). Before each experiment, a frozen microbial sample was spread on trypcase soy agar plate (Biomérieux, Crapone, France), or Chocolate agar plate (Biomérieux) for *H. influenzae*, and incubated at 36°C under aerobiosis or under 5% CO_2 for 16 h. For the yeast, frozen sample was spread on Sabouraud agar plate and incubated at 30°C under aerobiosis for 16 h. From this first preculture, a second preculture was made by transfer microbial strains on a new agar plate and incubated at 36°C (or 30°C) for 16 h. The second and the third precultures were used for the test. Suspensions were prepared as indicated in JIS Z 2801 in diluted Nutrient Broth (dilution ranging from 1/10 to 1/500 according to the bacteria viability control in assay conditions).

Viral strains and growth conditions

Considering their implication in human transmission, we tested Influenza virus A (H1N1) ATCC-VR-1520 (receiving cells: Raw-264-7, TIB-71) and Herpes virus type 1 (HSV-1) ATCC-VR-1383 (receiving cells: VERO, ATCC-

CCL-81). Cells and viruses are preserved following the standard recommendations (NF EN 14476 2015) in Eagles's minimal essential medium (EMEM) supplemented with 0·125% bovine serum albumin (BSA) for H1N1 or supplemented with 2% foetal bovine serum (FBS) for HSV-1.

The suspension titre was checked using the KARBER-SPAERMAN method calculating the negative logarithm of 50% endpoint (lgDICT50) by the following formula:

$$\text{lgDICT50} = \text{negative logarithm of the highest concentration of virus} - [(\text{sum of \% affected to each dilution}/100 - 0.5) \times (\text{log dilution})].$$

Microsphere description and integration into materials

The patented metal oxide microspheres are synthesized by a patented process of Pylote SA via a one-step "clean-tech" manufacturing process, called the Pyrolyse Pulvérisée® (Marchin 2015a, 2015b).

These ceramic particles were previously characterized: they have a coefficient of sphericity higher or equal to 0·75 and have a micrometer and a narrow distribution size (Marchin 2015a). These microspheres, on the basis of the technology PYCLEAR™ PROTECTION (microspheres not nanospheres) are listed as authorized additives for pharmaceutical containers in European, United States and Japanese Pharmacopeia and have a food certificate of compliance (Commission Regulation CE No 10/2011, FDA CFR 21 part). The introduced dose (0·1 to 2·5%) is within the authorized limits.

The technology of Pyrolyse Pulvérisée® is characterized by an ultra-fast thermal process (high-temperature for a few seconds) which does not allow the particles to densify totally. The latter remain porous with a specific surface of about 15 $m^2\ g^{-1}$ for microspheres. The technology of Pyrolyse Pulvérisée® leads a phenomenon of tempering and leads to oxides being not completely stoichiometric on-surface, creating surface defects such as interstitial ions and oxygen vacancies, very favourable to the production of reactive species.

Microspheres are directly integrated to final product materials by using the existing manufacturing process. Microsphere-added plastic plates of 100 cm^2 (10 cm × 10 cm) with a thickness of 2 mm were produced by conventional injection molding with the usual parameters for PolyEthylene (PE) and PolyPropylene (PP) polymers. PP films were produced by extrusion. The observation of microsphere incorporation in materials was performed by Scanning Electron Microscopy (JSM-6490, JEOL) after acid treatment. The average distance between two microspheres into materials was analysed by ImageJ software.

Control plastic plates (without microsphere incorporation) have been produced with same plastic raw materials batches, by same equipment process.

Antimicrobial surface activity testing

Tests were performed in the conditions of JIS Z 2801 (2010): temperature (35 ± 1°C), relative humidity (RH > 90%), inoculum size (10^4 units per cm^2), surface contact (recovering film for hydrophobic surface) and at least contact time (24 h).

Preparation of test plastic coupons

Plastic coupons of 25 cm^2 (5 cm × 5 cm) were cut from microsphere-added and control plastic plates of 100 cm^2 previously obtained. For each assay, six untreated coupons (C0 and C24h) and three treated coupons (A24h) were prepared. They were used after alcohol immersion for 10s followed by sterile distilled water rinsing and air drying.

Preparation of microbial suspensions and inoculation of test plastic coupons

Bacterial and yeast suspensions adjusted to $2{\cdot}10^8$ cells ml^{-1} (OD at 640 nm) were diluted to obtain a concentration that is between $2{\cdot}10^5$ cells ml^{-1} and $1{\cdot}10^6$ cells ml^{-1}. Dilutions were performed; thanks to a diluted Nutrient Broth (1/500 or 1/250; 1/10 for *S. pyogenes* for viability preservation).

Viral suspensions were diluted to obtain a concentration, that is between 5·4 lg DICT50 and 6 lg DICT50 in EMEM supplemented with 0·125% BSA for H1N1 or supplemented with 2% FBS for HSV-1.

The diluted suspension was then spread on the alloy surface (25 $\mu l\ cm^{-2}$) and recovered with a sterile polyethylene film that measures 4 cm × 4 cm (16 cm^2) as indicated for hydrophobic surfaces.

Incubation and microbial recovery from test coupons

Test coupons were then placed at 35 ± 1°C with RH>90% during the defined contact time (24 h). Considering viability loss for *B. catarrhalis* and *H. influenza* in the JIS Z 2801 standard conditions, contact time was reduced, respectively, to 4 h and 1 h (concentration on control coupons higher than 2 log after incubation).

At the end of the defined contact time, the residual micro-organisms were recovered by deposition of 10 ml of Soybean Casein Digest broth with Lecithin and Polyoxyethylene sorbitan monooleate (SCDLP, JIS Z 2801) with sterile beads, or cell scrapers (for viruses), on the tested surface and vortexing the mixture. Ten-fold dilutions were performed and 1 ml of each was incorporated in two trypcase soy agar (or Chocolate agar for *H.*

influenzae) Petri dishes for CFU numeration after 24–48 h incubation at 36 ± 1°C or in two Sabouraud agar Petri dishes for CFU numeration after 24–48 h incubation at 30°C for *C. albicans* (A24h) residual viral particles were numerated according to EN 14476 (2015). CFU counts (Colony Forming Units or Units for viruses) were transferred to CFU per cm^2 and then to log CFU per cm^2. According to JIS Z 2801 standard, the quantification limit is <1 CFU/surface (not any colony formations in any agar plate), corresponding to <0·63 CFU per cm^2 (0·00 log CFU per cm^2).

Controls (C0 and C24h) were performed using coupons without microsphere incorporation (C) to check the lack of antimicrobial activity and to calculate the log reduction.

Validation of each assay was checked according to the indication of JIS Z 2801 standard, considering that the logarithmic value of the number of viable bacteria recovered immediately after inoculation (T0) from the untreated test coupons shall satisfy the following equation:

$$(\mathrm{Vmax} - \mathrm{Vmin})/\mathrm{Vmean} \leq 0.2$$

where, Vmax is the maximum logarithmic number of viable bacteria; Vmin is the minimum logarithmic number of viable bacteria; Vmean is the average of logarithmic number of viable bacteria of three untreated test coupons.

Antimicrobial surface activity calculation

Antimicrobial activity (R) was calculated by using the following equation:

$$R = (\mathrm{C24h\text{-}C0})\text{-}(\mathrm{A24h\text{-}C0}) = \mathrm{C24h\text{-}A24h}$$

where, R is the logarithm reduction; C0 is the average of logarithm numbers (CFU per cm^2 or lgDICT50 for viruses) of viable micro-organisms recovered from the untreated coupons (controls) immediately after inoculation; C24h is the average of logarithm numbers (CFU per cm^2 or lgDICT50 for viruses) of viable micro-organisms recovered from the untreated coupons (controls) after 24 h; A24h is the average of logarithm numbers (CFU per cm^2 or lgDICT50 for viruses) of viable micro-organisms recovered from the treated coupons (microsphere-added) after 24 h.

C4h-A4h and C1h-A1h were considered, respectively, for *B. catarrhalis* and *H. influenzae*.

Results are expressed as means ± standard deviations (SD) of at least three independent experiments.

Antimicrobial surface activity characterization

Antimicrobial surface activity tests, as previously described, were performed in different conditions in an attempt to characterize the antimicrobial properties of the innovative process of homogeneous incorporation of metal oxide microspheres into materials.

Spectrum of activity

In a first step, the antimicrobial activity of the microsphere-added (2·5% w/w) surfaces was determined against the two bacteria recommended by the JIS Z 2801 standard (*E. coli* CIP 53·126 and *S. aureus* CIP 53·156) and also against two reference microbial strains of the European and United States Pharmacopoeia, *P. aeruginosa* CIP 82·118 and *C. albicans* DSM 1386. Then, the spectrum was expanded to other bacteria, antibiotic-resistant or not, as well as to viruses.

Impact of contact time

Antimicrobial activity assays were then carried out by varying the contact time (0·5, 1, 2, 4, 6, 24 h) between the plastic surfaces and the micro-organism *E. coli* CIP 53·126 in order to determine the minimum time required to obtain a significant effect.

Effect of microsphere concentration and plastic matrix

Tests were performed against *E. coli* CIP 53·126 using different composition and form of plastic surfaces (PE, PP and PP film) containing various concentrations of microspheres (0·5, 1·5, 2·5% w/w).

Effect of storage time and temperature

After determination of the antibacterial surface activity of recently prepared PE coupons containing 2·5% w/w of microspheres on *E. coli* CIP 53·126, same tests were performed on the same plastic coupons stored for different times in different temperature and relative humidity (RH) conditions, i.e. 6 months at 40°C/75% RH and 12 months at 25°C/60% RH corresponding, respectively, to accelerated and long-term stability studies conditions of the International Conference on Harmonization (ICH) Q1A(R2) guideline, and also 50 months at room temperature.

Simulation of use

Finally, the maintenance of the antimicrobial surface activity of the microsphere-added plastic surfaces after contact with commonly detergents-disinfectants used in health establishments was checked by exposing 2·5% w/w PE coupons to the selected products twice a day for 10 days, then measuring the antimicrobial activity against *E. coli* CIP 53·126 according to the JIS Z 2801 standard. The detergents-disinfectants tested were selected for their frequency of use and were diluted at the recommended doses of use in hospitals: Sodium hypochlorite solution (NaClO) at 0·9 %, Surfanios Premium (51 mg g^{-1} of N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine and

25 mg g^{-1} of didecyldimethylammonium chlorure, Laboratoires Anios®) diluted at 0·25%, and Anioxy-Spray WS (50 mg g^{-1} of hydrogen peroxide and 91·6 mg g^{-1} of ethanol, Laboratoires Anios®), ready-to-use product.

Statistical analysis

Student *t*-test was used to calculate the significance of the difference between the mean log reduction of coupons with microspheres according to the conditions and those of the controls (coupons without microspheres). Following the JIS Z 2801 standard test conditions, the number of independent experiments performed for each assay corresponds to $n = 3$, except for *E. coli* and *S. aureus* screening testing for which $n = 6$. Statistically significant values were defined as $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ and $^{****}P < 0{\cdot}0001$.

Results

Microsphere incorporation in materials

The incorporation of microspheres in materials was checked by observation with a scanning electron microscope. Particles are spherical and initially totally unagglomerated, which allows a very good dispersal of particles in the material (PE for example) as it is shown in Fig. 1. The distance between two microspheres is between 0·2 and 1 μm.

Antimicrobial surface activity: spectrum of activity

Preliminary assays were performed on PE coupons according to the JIS Z 2801 standard, on *E. coli* CIP 53·126 and *S. aureus* CIP 53·156, and on two other referenced microbial strains *P. aeruginosa* CIP 82·118 and *C. albicans* DSM 1386 (Table 1).

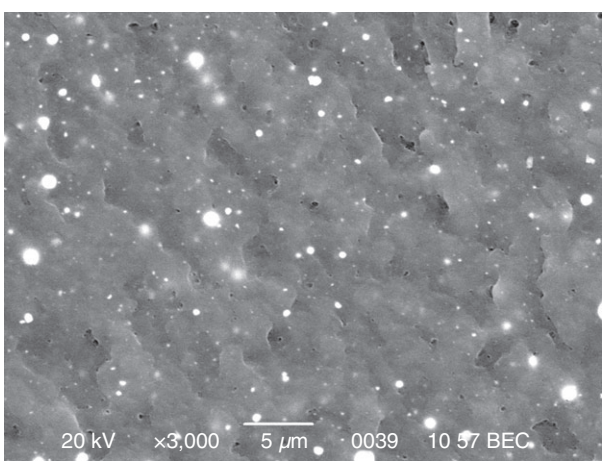

Figure 1 Metal oxide microspheres dispersed inside polyethylene plastic after acid attack. Microsphere content 2·5% w/w, Scanning Electron Microscope (JEOL JSM-6490, CEMES, 2015).

The PE (2·5% w/w microspheres) coupons of antimicrobial surface are characterized by a very high and significant activity, without any bacteria recovered on treated coupons, for the two Gram negative bacteria tested *E. coli* CIP 53·126 and *P. aeruginosa* CIP 82·118 ($P < 0{\cdot}0001$) and for *S. aureus* CIP 53·156 ($P < 0{\cdot}0001$). Antimicrobial activity against *C. albicans* DSM 1386 was also significant with about 1 log reduction ($P < 0{\cdot}01$).

Given the activity observed on these microbial reference strains, the antimicrobial spectra was swiped considering other microbial species, including bacteria expressing acquired mechanisms of resistance (*S. aureus* metiR, *E. coli* ESBL) and viruses (Table 2). The surfaces are able to reduce significantly the population of all the tested bacteria, including resistant ones. The virucidal activity on HSV-1 and H1N1 was also highly significant ($P < 0{\cdot}0001$) with more than 2 log reduction.

Antimicrobial surface activity according to the contact time

To better explore the bactericidal activity of the microspheres, experiments were performed by changing the contact time between the PE (2·5% w/w microsphere-added or not) surfaces and *E. coli* CIP 53·126 (Fig. 2). Microsphere-added coupons are able to reduce microbial population in a time-dependent manner with a decrease of 0·89 log, 2·18 log and more than 6·21 log after respectively 4, 6 and 24 h of contact.

Antimicrobial surface activity according to the microsphere concentration and the matrix

Then, the modification of bactericidal activity level was defined by using PE surfaces containing 3 concentrations of microspheres: 0·5%, 1·5% and 2·5% w/w (Fig. 3). A significant dose-dependent effect was noted with the most important result concerning the efficiency preservation at the lower concentration (0·5% w/w) tested ($P < 0{\cdot}0001$).

To complete these experiments, assays were also performed on *E. coli* CIP 53·126 according to the polymer: PE *vs* PP or PP film containing or not 2·5% w/w microspheres (Table 3). In such conditions, antimicrobial activity was preserved whatever the matrix with more than 6 log CFU per cm^2 reduction for treated PP coupons and treated PP film ($P < 0{\cdot}0001$).

Maintenance of the antimicrobial surface activity

The preservation of the antimicrobial activity was first analysed on PE coupons stored for different times in

Table 1 Log CFU numerations and log reductions per cm^2 after 0 h (control C0) and 24 h (control C24h and assay A24) of contact with PE coupons (2·5% w/w microsphere-added or not)

Strains	C0	C24h	A24h	Log reduction
S. aureus CIP 53·156	3·85 ± 0·02	3·79 ± 0·16	0·00 ± 0·00	>3·79 ± 0·13****
E. coli CIP 53·126	3·95 ± 0·03	>6·27 ± 0·00	0·00 ± 0·00	>6·27 ± 0·00****
P. aeruginosa CIP 82·118	4·05 ± 0·02	4·15 ± 0·09	0·00 ± 0·00	>4·15 ± 0·07***
C. albicans DSM 1386	3·96 ± 0·07	4·57 ± 0·05	3·60 ± 0·20	0·97 ± 0·17**

$^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

Results are expressed as means and SD of six independent experiments ($n = 6$) for *Staphylococcus aureus* and *Escherichia coli* or three independent experiments ($n = 3$) for *Pseudomonas aeruginosa* and *Candida albicans*.

Table 2 Log CFU per cm^2 or lgDICT50 for viruses and log reductions after 0 h (control C0) and 24 h (control C24h and assay A24h) of contact with PP coupons (microsphere-added or not)

Strains	C0	C24h	A24h	Log reduction
S. epidermidis CIP 68·21	3·63 ± 0·04	2·06 ± 0·39	0·00 ± 0·00	>2·06 ± 0·32***
S. pyogenes CIP 56·41T	3·78 ± 0·00	2·94 ± 0·09	0·03 ± 0·06	2·91 ± 0·08****
L. monocytogenes CIP 82·110T	4·16 ± 0·04	4·21 ± 0·11	0·00 ± 0·00	>4·21 ± 0·09****
S. enterica CIP 60·62T	3·75 ± 0·02	5·84 ± 0·05	0·00 ± 0·00	>5·84 ± 0·04****
S. aureus ATCC 33591 metiR	4·05 ± 0·02	3·01 ± 0·29	0·00 ± 0·00	>3·01 ± 0·24****
E. coli ESBL	3·83 ± 0·02	5·76 ± 0·04	1·93 ± 0·47	3·83 ± 0·39***
HSV-1	5·10 ± 0·10	6·03 ± 0·06	3·83 ± 0·12	2·20 ± 0·11****
H1N1	5·33 ± 0·15	5·03 ± 0·31	2·43 ± 0·16	2·60 ± 0·27***
Strains	C0	C4h	A4h	Log reduction
B. catarrhalis CIP 73·21T	4·11 ± 0·09	2·18 ± 0·28	0·00 ± 0·00	>2·18 ± 0·23***
Strains	C0	C1h	A1h	Log reduction
H. influenzae CIP 102514T	3·75 ± 0·06	3·19 ± 0·04	1·67 ± 0·07	1·52 ± 0·06****

$^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

Time of contact for *Branhamella catarrhalis* and *Haemophilus influenzae* was reduced to 4 h and 1 h, respectively. Results are expressed as means and SD of three independent experiments ($n = 3$).

different temperature and relative humidity conditions. Whatever storage times and conditions, antimicrobial activity for the microsphere-added surfaces was maintained (Table 4), even for coupons tested 50 months after their manufacture for which the reduction of the population for *E. coli* is higher than 6·12 log ($P < 0{\cdot}0001$).

PE coupons were also exposed to detergents and disinfectants twice a day for 10 days and then analysed for their antimicrobial activity against *E. coli* CIP 53·126. For all the products tested, no CFU is harvested after 24 h of contact with treated microsphere-added coupons (Table 5). For Surfanios Premium treatment, the observed decrease in the antimicrobial activity, i.e. more than 4·41 log whereas it was more than 6 log for unexposed coupons, may be due to a persistence of the disinfectant on the control surfaces (reduced value observed for C24h) and not to a reduction of the microsphere efficiency.

Discussion

Antimicrobial surfaces are considered as a new tool for the management of microbial contamination in many fields, including health, food and industry. Many studies on surfaces deal with nonantibiotic molecules with a focus on the active compounds release and the toxicity risk over a threshold level. As an example, surfaces with high concentrations of implanted ions showed high rates of dissolution into the surrounding medium even with nanoparticles (Furno *et al.* 2004; Harrasser *et al.* 2015). Gorzelanny *et al.* (2016) observed a fast and transient release of silver ions from diamond-like carbon implants enriched in silver nanoparticles able to reduce the growth of *S. aureus* et *S. epidermidis* on the surface. They linked this release to the biocompatible behaviour of the implants, ensuring growth of mammalian cells. Despite this, the mechanism of activity of silver is achieved by

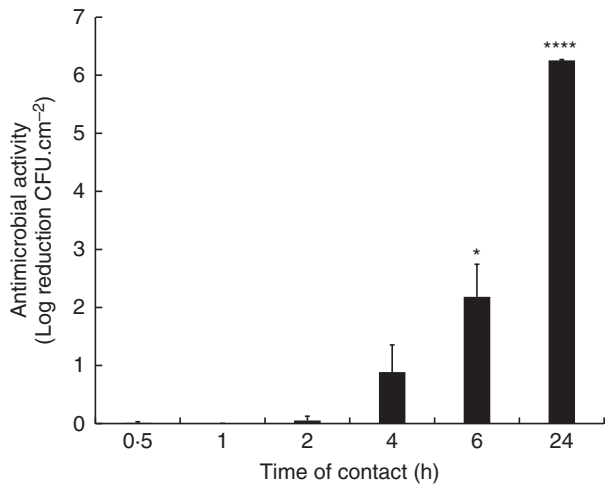

Figure 2 Antimicrobial activity in Log CFU reduction per cm^2 of *Escherichia coli* CIP 53·126 after different times of contact with PE coupons (2·5% w/w microsphere-added or not). $*P < 0·05$; $****P < 0·0001$. Results are expressed as means and SD of three independent experiments ($n = 3$).

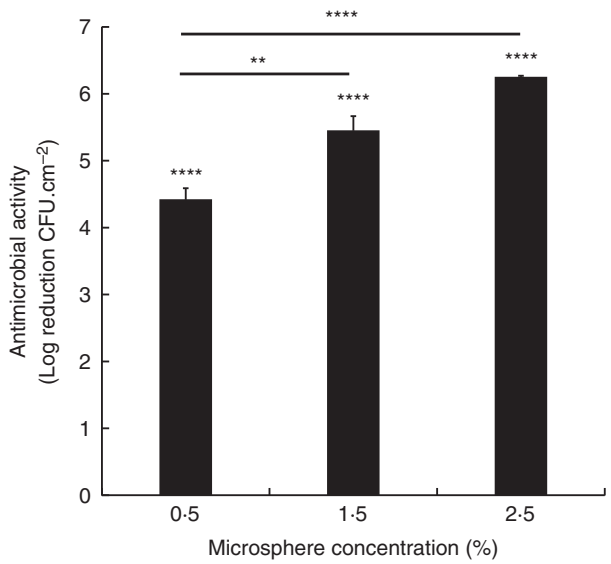

Figure 3 Antimicrobial activity in Log CFU reduction per cm^2 of *Escherichia coli* CIP 53·126 after 24 h of contact with PE coupons containing different concentration of microspheres (0 to 2·5% w/w). $**P < 0·01$; $****P < 0·0001$. Results are expressed as means and SD of three independent experiments ($n = 3$).

binding to microbial membranes, enzymes and nucleic acids, leading to the inhibition of the respiratory chain (Gosheger *et al.* 2004). This nonspecific mode of action motivates caution concerning the long-term toxicity risk for human and environment.

In opposition, compounds with oxidative properties, like zing (ZnO) and magnesium (MgO) oxides, may be considered of interest because of the rapid biodegradation of active molecules (Pasquet *et al.* 2014, 2015; He *et al.* 2016). Antimicrobial activity of these metal oxides is not completely illuminated and still controversial (Sirelkhatim *et al.* 2015). However, Fiedot *et al.* (2017) have recently confirmed that the generation of free radicals, which occurs on the surface of crystalline ZnO, was crucial for its antimicrobial activity. According to Applerot *et al.* (2012) and Brown *et al.* (1999), the surface of numerous oxides (ZnO, CuO, MgO, Ln_2O_3) has a character electro donor which leads, in the contact of the water, to the formation of Reactive Oxygen Species (ROS), mostly OH° radicals. This catalytic mechanism, not photo-activated, depends on the presence of specific defects on the metal oxide surface such as oxygen vacancies (Applerot *et al.* 2009). These hydroxyl radicals formed on particles surface are strongly oxidizing and destroy bacteria when particles and/or reactive species are in direct contact with micro-organisms (Applerot *et al.* 2009). This effect occurs in the immediate neighbourhood of the particle (dozens of nanometers at the most) because the life cycle of the hydroxyl radicals is very short (nanosecond).

On the other hand, there are also some queries within the spectrum of activity of metal oxides requiring deep mechanism explanations (Sirelkhatim *et al.* 2015). Previous studies reported that Gram positive (*S. aureus*, *S. epidermidis* and *B. subtilis*) bacteria were more sensitive to ZnO particles than Gram negative (*E. coli* and *P. aeruginosa*) bacteria (Azam *et al.* 2012; Fiedot *et al.* 2017). For these authors, the differences in the cell wall structure play a significant role. Gram positive bacteria have a much thicker cell wall without outer membrane compared to Gram negative bacteria, which results in a decreased susceptibility to the membrane damage induces by ZnO particles. However, as observed by Pasquet *et al.* (2014), there is no significant difference in susceptibility between the Gram positive and Gram negative bacteria in

Table 3 Log CFU numerations and log reductions per cm^2 of *Escherichia coli* CIP 53·126 after 0 h (control C0) and 24 h (control C24h and assay A24h) of contact according to the matrix (2·5% w/w microsphere-added or not)

Material	C0	C24h	A24h	Log reduction
PE coupons	3·95 ± 0·03	>6·27 ± 0·00	0·00 ± 0·00	> 6·27 ± 0·00****
PP coupons	3·99 ± 0·03	6·32 ± 0·04	0·00 ± 0·00	> 6·32 ± 0·03****
PP films	3·99 ± 0·03	6·28 ± 0·03	0·00 ± 0·00	> 6·28 ± 0·02****

PE, polyethylene; PP, polypropylene. $****P < 0·0001$. Results are expressed as means and SD of three independent experiments ($n = 3$).

Table 4 Log CFU numerations and log reductions per cm^2 of *Escherichia coli* CIP 53·126 after 0 h (control C0) and 24 h (control C24h and assay A24h) of contact with PE coupons (2·5% w/w microsphere-added or not) placed in different storage conditions

Storage conditions	C0	C24h	A24h	Log reduction
6 months, 40°C/75%RH	3·96 ± 0·14	6·09 ± 0·07	0·00 ± 0·00	> 6·09 ± 0·05****
12 months, 25°C/60%RH	4·04 ± 0·02	5·99 ± 0·16	0·70 ± 1·22	5·29 ± 1·00**
50 months, room temp.	4·10 ± 0·02	6·12 ± 0·06	0·00 ± 0·00	> 6·12 ± 0·04****

$^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$. Results are expressed as means and SD of three independent experiments ($n = 3$).

Table 5 Log CFU numerations and log reductions per cm^2 of *Escherichia coli* CIP 53·126 after 0 h (control C0) and 24 h (control C24h and assay A24h) of contact with PE coupons (2·5% w/w microsphere-added or not) exposed to different detergents-disinfectants twice a day for 10 days

Treatment	C0	C24h	A24h	Log reduction
Untreated	3·95 ± 0·03	>6·27 ± 0·00	0·00 ± 0·00	>6·27 ± 0·00****
NaClO	4·02 ± 0·03	>6·24 ± 0·03	0·00 ± 0·00	>6·24 ± 0·02****
Surfanios Premium	3·99 ± 0·03	4·41 ± 1·11	0·00 ± 0·00	>4·41 ± 0·90**
Anioxy-Spray	4·02 ± 0·02	6·34 ± 0·03	0·00 ± 0·00	>6·34 ± 0·03****

$^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$. Results are expressed as means and SD of three independent experiments ($n = 3$).

our study. As Nair *et al.* (2009) suggested, these results confirmed that the antibacterial action mechanism of ZnO particles mainly depends on the ROS generation because the membranes of Gram positive and Gram negative bacteria are equally permeable to ROS. Although metal oxides particles have been widely examined for their antibacterial properties, there are very few studies regarding their antifungal activity (Sawai and Yoshikawa 2004; Pasquet *et al.* 2014). Our results are in agreement with those obtained by these authors with a weak activity of ZnO against *C. albicans*. There are no studies regarding the antiviral activity of metal oxides. Additional analyses need to be done to clarify the mechanism and the interesting antiviral activity observed in our study.

ZnO has multiple applications such as preservative agent, but only a few concerned antimicrobial surfaces (de Rancourt *et al.* 2013; Dobrucka 2014; Salarbashi *et al.* 2016). Problems encountered are linked to the hydrophily of the particles and, when used under nanoparticles, their agglomeration and nonhomogeneous repartition, meaning nonhomogeneous availability of active molecules. On the other hand, the antibacterial activity described for ZnO and MgO nanoparticles may appear limited if we think only of the test methods (agar diffusion technique or broth dilution method) which are unable to demonstrate only inhibition of growth and sometimes of adhesion (Jin and He 2011; Tang and Lv 2014; Elkady *et al.* 2015; McGuffie *et al.* 2016). In comparison, our assays with incorporated microspheres of metal oxides into plastic materials led to the demonstration of a lethal effect on bacteria and viruses observed in the same range of concentrations (since 0·5% (w/w)).

The high level of antimicrobial activity demonstrated in this study was linked to two main important characteristics of the added surfaces. First, particles are spherical and initially totally unagglomerated, which allows a very good dispersal of particles into materials (plastic for example) as it is shown on Fig. 1. The antibacterial effect of particles being a short distance effect, every bacterium in the assay conditions described, was expected to touch or be close to one particle and/or one ROS generated by this particle. Indeed, Fig. 1 shows also that the surface average distance between two particles is between 0·2 and 1 μm which is the same order of magnitude than the average size of bacteria (1 μm). In 2013, Silvestre *et al.* showed less effective antibacterial activity (less than 2 log) using nanometric ZnO (d_{50} = 490 nm, Sw = 13·5 $m^2\ g^{-1}$) embedded in isotactic PolyPropylene (iPP). A careful observation of these particles by electron scanning microscopy clearly shows that used nanoparticles are strongly agglomerated inside the polymer matrix (Silvestre *et al.* 2013). Even if theoretically, nanoparticles should be more efficient than micronic ones, practically, it is counter-productive to use nanoparticles if you are not able to disperse them effectively inside the plastic matrix. This problem is especially widespread with hydrophobic polymers which are not compatible with hydrophilic nanoparticles, such as ZnO and MgO. At the same time, nanoscale might be considered responsible for a part of the human cell damages (direct contact and internalization of nanoparticles) as demonstrated for TiO_2 nanoparticles in the dark (Gogniat *et al.* 2006; Liu *et al.* 2007; Verdier *et al.* 2014). The nanoscale's toxicity and ecotoxicity are thus being questioned.

However, the exceptional antimicrobial activity reported for these microparticles cannot be explained only by the dispersion quality. Indeed, other tests reported by authors using commercial dense micronic particles never give antibacterial activity higher than 2 log in the range of 0·1 to 3·0% w/w (Silvestre *et al.* 2013). With a specific surface area about 15 $m^2\ g^{-1}$ (approximately 15 times as important as the commercial microparticles of 1 μm of diameter), these microparticles should produce as much reactive oxygen species as nanometric ones. Indeed, 15 $m^2\ g^{-1}$ is usually met on nanoparticles of dozens of nanometers of diameter, and not on dense micron size particles. As Gumy *et al.* (2005), we previously showed the importance of contact optimization between another nonorganic oxide, TiO_2 and bacteria (Verdier *et al.* 2014). Among the parameters involved in the antimicrobial activity spectrum and level, we can find the nature and morphological structures of particles (Elkady *et al.* 2015) with potent direct physical effect of nanometric ones (Verdier *et al.* 2014) and the surface morphology (Oh *et al.* 2015).

The most important property of the system was certainly its ability to return exactly to its initial state without creation or destruction of material on the catalyst (oxide). As any catalytic phenomenon, the production of on-surface reactive species of the particles of oxide can work in theory infinitely as long as reactive species (H_2O and O_2) are supplied and the catalyst (microspheres) is not being consumed. Our assays demonstrated this activity is preserved for 50 months after manufacturing which was also in line with the proof that the catalyst was not released from the material (data nonshown) and confirms the interest of such microspheres' addition to material in the management of surface contamination.

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Conflict of Interest

A part of the financial support to conduct this study was provided by Pylote SA (Dremil-Lafage, France). C. Feuillolay, L. Haddioui and C. Roques declare that they have no conflict of interest concerning this article. They respectively made and validated the tests, wrote the protocols and the article after analysing the results. Marc Verelst was involved in the physico-chemical characterization and the article writing. L. Marchin as the Manager of Pylote and A. Furiga in charge of Pylote R&D, validated the protocols, prepared the samples and approved the article.

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