Diamond & Related Materials 81 (2018) 118–126

Contents lists available at ScienceDirect

Diamond & Related Materials

journal homepage: www.elsevier.com/locate/diamond

Diamond like carbon Ag nanocomposites as a control measure against *Campylobacter jejuni* and *Listeria monocytogenes* on food preparation surfaces

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ARTICLE INFO

Keywords:
Campylobacter jejuni
Listeria monocytogenes
DLC Ag nanocomposites

ABSTRACT

The effect of thin (5 nm) and thick (40 nm) silver layers and diamond like carbon nanocomposites with embedded Ag nanoparticles (DLC:Ag) against two reference strains of *C. jejuni* NCTC 11168 and *L. monocytogenes* ATCC 7644 were evaluated in this study. DLC:Ag film contained 22 at.% Ag. Silver nanoparticle size measured by transmission electron microscope was in the 5–10 nm range. Ag layers and DLC:Ag nanocomposites were deposited employing unbalanced reactive magnetron sputtering on crystalline silicon wafers. *C. jejuni* and *L. monocytogenes* numbers were counted by culture-based enumeration on selective agars and quantitative real-time PCR (qPCR) including staining with propidium monoazide (PMA). It was determined, that DLC:Ag film was the most efficient coating in the reduction of *C. jejuni* and *L. monocytogenes* numbers. Culture-based enumeration revealed that *C. jejuni* numbers were reduced by an average of 4.06 $\log_{10}$CFU/ml after 15 min and 3.61 $\log_{10}$CFU/ml after 30 min on DLC:Ag coated silicon wafers in comparison to control samples ($P \leq 0.05$). *L. monocytogenes* was not detected on DLC:Ag samples after 24 h of exposure ($P \leq 0.05$). PMA-qPCR showed that *C. jejuni* and *L. monocytogenes* affected by DLC:Ag antimicrobial surface showed a reduced ability to grow on culture media, but maintained viability during the whole experiment. Nonetheless, DLC:Ag antimicrobial surface could be further considered for the reduction of cross-contamination risk from food preparation surfaces due to their contamination with *C. jejuni* and *L. monocytogenes* in domestic and commercial kitchens or food establishments.

1. Introduction

C. jejuni pose a great risk to human health as the main cause for bacterial human gastroenteritis in the European Union [1]. Poultry reservoir is considered to be the main source of *Campylobacter* infections in humans [1–3]. Significantly lower numbers are reported for *Listeria* infections, that are usually related to consumption of fresh meat, raw milk or raw milk products and poultry [4–6], however *L. monocytogenes* is responsible for a high mortality rate of infected patients, especially affecting YOPI (young, old, pregnant or immunodeficient) groups [1,5]. Up to now, there are no effective measures for the total elimination of *C. jejuni* and *L. monocytogenes* on animal carcasses or the food environment. As a result, bacteria spread from contaminated carcasses and slaughterhouse equipment to processed meat products at the food processing establishments [4,7].

One way to protect the final product from contamination with pathogenic bacteria is to use physical and chemical decontamination measures in the slaughterhouse environment. Therefore, cleaning of slaughterhouse equipment with high-pressure water, sodium hydroxide and potassium hydroxide combination is usually followed by the application of various disinfectants like quaternary ammonium compounds combined with glutaraldehyde, poly (hexamethylene biguanide) hydrochloride and foam alkaline detergents [6–9]. However, they are not always efficient in the reduction of *C. jejuni* and *L. monocytogenes* due to bacteria resistance to disinfectants [6,10,11].

The coating of antimicrobial surfaces with silver could demonstrate a prolonged effect against pathogenic bacteria and low cytotoxicity to the final product [12]. Silver is known for antimicrobial properties against Gram-negative and Gram-positive bacteria [13] and silver nanoparticles, ranging in size from 1 to 100 nm, are efficient in the reduction of pathogenic bacteria like *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholera* and *Streptococcus sanguinis* [14–17]. The reduction effect is based on an increased contact surface between silver nanoparticles and bacteria cell [13,18,19]. Additionally, nanoparticles in the

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https://doi.org/10.1016/j.diamond.2017.12.007
Received 7 October 2017; Received in revised form 15 November 2017; Accepted 4 December 2017
Available online 06 December 2017
0925-9635/

diameter lower than 10 nm have a better reactivity during the interaction with target bacteria as more valence electrons are available for surface-adsorbent bonds [20,21]. On the other hand, the application of silver nanoparticles in colloidal form usually has a short antimicrobial effect as silver ions are released quickly [22]. However, the antimicrobial effect could be prolonged by incorporating Ag nanoparticles into robust materials like diamond like carbon (DLC), leading to higher mechanical resistance [23,24,25]. Silver nanoparticles introduced by magnetron sputtering into DLC, release silver ions slowly and enhance the antimicrobial effect of this kind of surface [26]. Additionally, the DLC by itself is biocompatible and robust, the released amount of silver from the robust matrix is time dependent [26] and the amount distributed on the surface of the food in contact is tolerant and harmless [24] or can be removed under washing and normal cooking conditions [21]. Interestingly, undoped DLC has also shown antibacterial properties against Gram-negative and Gram-positive bacteria [27]. Also, it was recently shown that DLC modified with germanium has an antimicrobial activity against *P. aeruginosa* bacteria as reduction of biofilm formation was observed [28]. Additionally, DLC containing tungsten, cobalt inhibited the growth of *Candida albicans*, *E. coli* and *P. aeruginosa*, however, DLC:Ag was the most effective between the tested metal nanoparticles containing DLC films [29].

It must be noted, that culturable *C. jejuni* are detected for up to 3 h on metallic surfaces and for longer time periods on wooden and plastic surfaces [30]. Nonetheless, this interval is sufficient for cross-contamination and infection [31,32]. In contrast, *L. monocytogenes* is more resistant to stressful environment conditions and may persist on various surfaces for months or even years [4,11,33].

Therefore, the aim of this study was to evaluate the potential of surfaces covered with diamond like carbon (DLC) nanocomposite with embedded silver nanoparticles (DLC:Ag) as a control measure against *C. jejuni* and *L. monocytogenes* on food preparation surfaces.

2. Materials and methods

2.1. Preparation and characterization of antimicrobial films

In the present study hydrogenated DLC:Ag films of 50 nm thickness containing 22 at.% of silver [34,35] were deposited by direct current (DC) unbalanced reactive magnetron sputtering of silver target (99.99% purity, Kurt J Lesker). The diameter of magnetron was 3 in. Monocrystalline silicon substrates were used. Mixture of the hydrocarbons (acetylene, C_2H_2) and argon (Ar) gas was used in the reactive magnetron sputtering. Ar gas flux was 80 sccm (standard cubic centimeters per minute) and C_2H_2 gas flux was 7.8 sccm.

Silver films of 5 nm (Ag1) and 40 nm (Ag2) thickness were deposited by the same DC unbalanced reactive magnetron sputtering of silver target for comparison purposes. In this case, only Ar gas with a flux of 80 sccm were introduced into the chamber. In all experiments substrate–target gap was set at 10 cm, magnetron target current was 0.1 A, base pressure was 5×10^{-4} Pa and work pressure was $(4 \pm 1) \times 10^{-1}$ Pa. Substrates were grounded, no additional bias was applied. Quartz microbalance was used to control the deposition rate of the films. Summary of the samples and deposition rates used is presented in Table 1.

The surface of the prepared samples was studied employing field emission scanning electron microscope (FE-SEM) FEI Quanta 200 FEG. Theoretical resolution of the SEM at 30 kV accelerating voltage is 1.2 nm. Raman scattering measurements were performed using a Raman microscope inVia (Renishaw). The excitation beam from a diode laser of 532 nm wavelength was focused on the sample using a 50 × objective (NA = 0.75, Leica). Laser power at the sample surface was 1.75 mW, integration time was 10 s, signal was accumulated 1 time. The Raman Stokes signal was dispersed with a diffraction grating (2400 grooves/mm) and data was recorded using a Peltier cooled charge-coupled device (CCD) detector (1024 × 256 pixels). This system yields a spectral resolution of about 1 cm^{-1}. Silicon was used to calibrate the Raman setup in both Raman wavenumber and spectral intensity.

Table 1
List of investigated samples with abbreviations further used in the text.

Sample	Abbreviation	Deposition rate
Surfaces tested for antimicrobial efficiency		
Diamond like carbon nanocomposite with Ag, 50 nm thickness	DLC:Ag	0.3 nm/s
Thin silver layer, 5 nm thickness	Ag1	0.3 nm/s
Thick silver layer, 40 nm thickness	Ag2	0.5 nm/s
Control surfaces		
Silicon	S	Bulk sample
Stainless steel	SS	Bulk sample
High density polyethylene	HDPE	Bulk sample

2.2. Preparation of inocula

C. jejuni NCTC 11168 (ATCC 700819) and *L. monocytogenes* ATCC 7644 reference strains were used in this experiment. Bacteria strains were stored at − 80 °C in BHI broth (Liofilchem, Roseto degli Abruzzi, Italy) containing 30% glycerol. *C. jejuni* was cultivated on blood agar base No. 2 (Liofilchem) containing 5% of horse blood at 37 °C for 48 h under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2). *L. monocytogenes* was grown on ALOA (Agar Listeria acc. to Ottaviani & Agosti, Biolife, Milano, Italy) with ALOA Enrichment Selective Supplements (Biolife) at 37 °C for 24 h under aerobic conditions. A 10 µl loop of grown bacteria was transferred to 1 ml of PBS solution (Oxoid, Basingstoke, England) and optical density was measured at 600 nm wavelength with a spectrophotometer in order to achieve *C. jejuni* and *L. monocytogenes* numbers of approximately 8.0 $\log_{10}$ CFU/ml.

2.3. Experimental design

Overall 108 wafers (1.5 × 1.5 cm) made of crystalline silicon (University Wafer, South Boston, USA), high density polyethylene (HDPE 500, Hendi, Rhenen, The Netherlands) and stainless steel type 304 (Bosinox, Kaunas, Lithuania) were prepared for the experiment. Before inoculation, wafers were kept at room temperature in the dark. Control wafers were cleaned and disinfected with 96% ethanol (Vilniaus degtinė, Vilnius, Lithuania). They were left to air-dry for 30 min before the inoculation of bacteria. Experimental crystalline silicon wafers were sterilised by heating at 170 °C temperature for 1 h, coated with DLC:Ag and silver layers and held under sterile conditions until the beginning of the experiments.

C. jejuni and *L. monocytogenes* bacterial suspensions were inoculated on 45 and 63 wafers, respectively, by spreading 15 µl of liquid with a pipette on the wafer surface. The volumes of bacterial suspension added to the wafer were counted according to Hedin et al. [36]. The volume of bacterial suspension was counted by multiplying 10 µl with the length of wafer expressed in centimeters (1.5 cm). No significant differences ($P \leq 0.05$) were determined between *C. jejuni* and *L. monocytogenes* numbers considering three separate repetitions.

Wafers made from crystalline silicon (S), high density polyethylene (HDPE) and stainless steel (SS) were chosen as control samples to determine if there is any difference between the survival of tested bacteria on different materials during certain periods of the experiments. *C. jejuni* survival was determined in three time intervals: after initial inoculation, after 15 min and after 30 min, while *L. monocytogenes* was tested after initial inoculation (0 h), 1 h, 4 h and 24 h, each in triplicate. Shorter time periods for *C. jejuni* were chosen to represent the average time when poultry meat is processed in domestic and commercial kitchens with a risk for cross-contamination, considering bacteria