Antibacterial efficacy of advanced silver-amorphous carbon coatings deposited using the pulsed dual cathodic arc technique

J. L. Endrino¹⁺, A. Anders², J.M. Albella¹, J. A Horton³, T. H. Horton³, P. R. Ayyalasomayajula⁴, M. Allen⁵

¹ Instituto de Ciencias de Materiales de Madrid, Madrid, Spain
² Lawrence Berkeley National Laboratory, Berkeley, California
³ SUNY Upstate Medical University, Syracuse, New York
⁴ Nano-Ram Technologies, Bangalore, India
⁵ Ohio State University, Columbus, Ohio

*E-mail: jlendrino@icmm.csic.es

Abstract. Amorphous carbon (a-C) also referred as diamond-like carbon (DLC) films are well known to be a biocompatible material with good chemical inertness; this makes it a strong candidate to be used as a matrix that embeds metallic elements with an antimicrobial effect. We have deposited a set of a-C:Ag films using a dual-cathode pulsed filtered cathodic arc source, the arc pulse frequency of the silver and graphite cathodes was controlled in order to obtain samples with various silver contents. In this study, we show the deposition of silver and carbon ions using this technique and analyze the advantages of incorporating silver into a-C by studying the antimicrobial properties against staphylococcus of samples deposited on Ti-6Al-4V coupons and evaluated using 24-well tissue culture plates.

Keywords. antibacterial, silver, filtered cathodic arc deposition, DLC, diamond-like carbon.

1. Introduction

Many orthopedic operations involve potentially contaminated surgical sites. Unfortunately, the use of orthopedic implants is associated with a relatively high incidence of infection. In addition to causing local pain, infections around surgical implants cause bone destruction that, ultimately, produce loosening of the implant and prevents the bone from healing. In an attempt to address this, advanced antibacterial coatings can be developed to prevent infections by decreasing the bacterial adhesion to the implant and that can also enhance bone growth around the implanted devices. DLC refers to an amorphous form of carbon in which the bonding is composed of a mixture of diamond type (sp³) and graphite type (sp²) hybridization. DLC has many properties in common with diamond, including high hardness, Young's modulus and chemical inertness. The material is of particular interest with regard to medical implants because it is highly biocompatible and can be deposited by physical vapor deposition (PVD) processes, which are done at much lower temperatures than chemical vapor deposited (CVD) diamond films. However carbon-based coatings do not possess any inherent antibacterial activity.
Metallic coatings such as silver can be effective in killing bacteria, but they can also produce a chronic inflammatory reaction that can lead to adverse tissue responses around a silver-coated device.

Table 1. Summary of some previous works in the literature related to antibacterial properties of modified DLC films.

| Authors                     | Material      | Deposition Process                                      | Main Results                                                                 |
|-----------------------------|---------------|---------------------------------------------------------|------------------------------------------------------------------------------|
| Endrino et al. [1]          | a-C:Ag, a-C:H-Ag | Plasma immersion ion implantation and deposition (PIII&D) | No adverse cell growth of osteoblast MC3T3 cells in both hydrogenated and non-hydrogenated DLC containing 5 at. % Ag but non-hydrogenated samples had higher cell viability. |
| Hauert et al. [2, 3]        | Ag/a-C:H      | PACVD and magnetron sputtering                          | 8 at.% Ag did not cause any change in cell growth rate of osteoblast MC3T3 cells but 16 at.% Ag decreased growth rate by a factor of two. |
| Ishihara et al. [4]         | F-DLC         | Reactive RF magnetron sputtering in mixed Ar, CH₄, CF₄ gas | Fluorine incorporation decreased the adhesion of Escherichia Coli bacteria to the DLC. |
| Katsikogianni et al. [5, 6] | Ag-DLC:F      | Atom beam/magnetron sputtering.                         | The adhesion of S. epidermidis bacteria to the Ag-DLC coating was greater than that for Ag but less than that observed for DLC coating. |
| Kwok et al. [7]             | Ag-DLC        | Pulsed filtered cathodic vacuum Arc                     | Samples deposited with very high Ag concentration on the surface (60-80 at.%). DLC-Ag films proved effective against Escherichia coli bacteria. |
| M.L Morrison et al. [8]    | Ag-DLC, Pt-DLC AgPt-DLC | Pulsed laser deposition (PLD) | Doping of diamond-like carbon with silver and platinum had increased antimicrobial efficacy against Staphylococcus warneri. |
| Narayan et al. [9]          | Ag-Pt-DLC     | Pulsed laser deposition (PLD)                          | Antimicrobial efficacy of DLC-Ag-Pt reported against Staphylococcus and Pseudomonas aeruginosa bacteria. |

Thus, silver-containing diamond-like carbons have attracted a lot of attention in the field of antibacterial coatings due also to promising antibacterial (see Table 1) and tribological properties [10]. Generally speaking, silver-containing DLC materials can be divided in two groups: hydrogenated and non-hydrogenated DLC, which is dependent on the deposition technique. In our recent work [1], we carried out a comparative set of depositions using either pulsed dual-cathode filtered cathodic vacuum arc (PDC-FCVA) to obtain non-hydrogenated samples and plasma based ion immersion implantation and deposition (PBIIID) using methane gas as precursor to obtain a hydrogenated matrix (see videos in Fig. 1). Our result concluded that the non-hydrogenated DLC matrix was better because they promoted higher cell viability of mouse osteoblastic cells and therefore from a biomedical point of view are a preferred choice for the preparation of antibacterial coatings.
2. Experimental

2.1 Deposition method

Amorphous carbon films doped with silver were prepared on glass disc substrates by PDC-FCVA. The deposition system consisted of a dual-cathode “triggerless” mini-gun designed to operate in pulsed mode [11]. Pulsed arc discharges on individual cathodes were triggered by a computer control system. The pulses used in the production of carbon plasma were 100 A and had a duration of 5 ms, while the metal plasma was made using pulses 1 ms long and a current of 700 A. The plasma stream produced by the source is injected into a 90-degree filter to remove most of the macroparticles which were formed during the cathodic arc process, the output of the filter can be observed in the videos of figures 1a and 1b. After exiting the filter, the plasma expanded through a homogenizer device composed of concentric magnets and allowed to have homogeneous thickness and composition in the substrates over an area of 80 cm². During the deposition of carbon pulses, the substrates were biased with negative 1 kV pulses that were 2 μs long with an off time between pulsed of 14 μs. The substrate was kept at ground potential during the deposition of the metal plasma (hence “species selective bias”) [12]. The pulse repetition rate was set at 2 pulses per second and the number of pulses per deposition was 5000. During the deposition, the substrate holder was rotated at a speed of 2 revolutions per minute (RPM). The residual gas pressure was typically in the $10^{-4}$ Pa range. The thicknesses of the deposited films ranged from 50 nm to 300 nm.

Figure 1. Videos of the deposition processes used to obtain non-hydrogenated and hydrogenated DLC-Ag films. Plasma homogenizer between filter output and rotating substrates was removed for illustrating purposes. (a) Video of pulsed filtered cathodic arc alternating graphite and silver pulses. (b) Video of plasma based ion implantation and deposition of methane simultaneously with pulsed filtered cathodic deposition of silver (process shown here only for comparative purposes). The violet plasma discharge around and behind the substrate holder indicates the continuous implantation from methane gas. Note: videos are already embedded in the pdf document. Adobe reader with adobe flash player is needed to watch the videos. Security settings should allow the embedded functionality.

2.2 Surface energy

A KRUSS DAS 100 instrument was used for contact angle measurements. This computer-controlled contact angle analyzer allowed us to measure static and dynamic contact angle along with surface tension and surface energy. The position of the sample stage can be precisely adjusted along the x-, y-, or z-axis. An optical system using variable intensity illumination controls the degree of backlighting. The optics can be tilted by up to 3° allowing us to choose the angle of view required for rough or uneven surfaces. The instrument contains a stepping motor controlled syringe system for precise and repeatable liquid drop formation and application. This insures that a reproducible drop volume is applied to the surface. The micro-syringe attachment facilitates rapid and precise formation of drop specimens. Following placement of a sessile drop, the syringe is easily swung from the field of view.
It will return to its exact working location for placement of the subsequent drop. Alternatively, it can remain at the working location, allowing continuously adding or withdrawing fluid from a sessile drop for the purpose of forming advancing or receding contact angles. After placing the water droplet, the contact angle was measured using the circular curve fit option of the software.

2.3 Antibacterial tests
Sterilized glass discs with the indicated a-C:Ag surface composition were transferred to 12-well polystyrene culture plates, and pre-treated in 1 ml DMEM media (DMEM is the abbreviation for Dulbecco/Vogt modified Eagle's (Harry Eagle) minimal essential medium) supplemented with 10% fetal calf serum (CellGro, Herndon, VA, USA) 24 hours at 37°C with agitation at 100 RPM. Following pre-treatment, 100 μl aliquots of the media were reserved for subsequent verification of initial sample sterility. No pre-existent microbial contamination was observed after 48 hours of incubation onto solid BHI agar.

Lyophilized cultures of *Staphylococcus aureus* (ATCC #25923, Manassas VA, USA) were revived in Brain-heart Infusion liquid media (BBL-BHI broth, Becton, Dickinson and Co., Sparks, MD, USA) for 6 hours at 37°C with agitation at 220 RPM. From this culture, 100 μl were applied to Brain Heart Infusion solid agar media (BBL-BHI agar, Becton, Dickinson and Co.) and incubated for 36 hours at 37°C. For each experiment, a single colony (~1.5 mm) was selected from the agar plate and dispersed in 12.5 ml Dulbecco’s modification of Eagle’s media supplemented with 10% fetal calf serum (DMEM/10%FCS). The DMEM/10% FCS cell suspension was allowed to grow 3-4 hours at 37°C with agitation at 220 RPM until a spectrophotometric optical density of approximately 0.5 AU was achieved at 670 nm wavelength. Subsequently, the suspension volume was adjusted with fresh media to normalize the inocula for each replicate experiment to 0.3 AU at 670 nm, yielding on approximately 1.64 x10^7 CFU/ml after incubating a 16-fold diluted suspension on BHI agar for 36 hours at 37°C.

The remaining media was aspirated following pre-treatment of the a-C:Ag discs, replaced with 1 ml of the DMEM/10% FCS cell suspension and incubated for 24 hours at 37°C with agitation at 100 RPM. After incubation, 500 μl of media from each well was collected, transferred to a sterile polypropylene microcentrifuge tube and stored briefly on ice. To quantify bacterial viability on solid media, 1 μl of the collected cell suspension was diluted into 1 ml sterile PBS, and from this dilution, 10 μl was spread over the surface of a BHI agar plate. After 24 hours of growth at 37°C, a colony number was estimated, and from the number of colonies used to express the number of viable cells number in colony forming units/μl (CFU). The remaining media was aspirated, and the discs were washed three times in sterile phosphate buffered saline (PBS, pH 7.4) to remove cells that were not adhered to the surface of the DLC:Ag discs. Following the final wash, the discs were stained for 15 minutes at room temperature in a solution of 10 μM Sytox-9 and 60 μM propidium iodide (BacLight Live/Dead Kit, Invitrogen, Carlsbad, CA, USA) dissolved in Phosphate buffered saline (PBS) as recommended in the manufacturer’s protocol. Subsequently, the discs were transferred to glass microscope slides and coverslipped with the supplied mounting oil. Digital micrographs were captured through a 100x oil-immersion objective under epifluorescent illumination using FITC (Ex.480nm/Em.535) or G2A (Ex.510nm/Em590nm) dichroic filter sets to respectively visualize Sytox-9 (green) or propidium iodide (red) stained cells. Corresponding monochromatic images were processed and merged using Image Pro Plus software (V4.0, Media Cybernetics, Silver Spring, MD, USA).

3. Results
3.1 Wettability
The ratio of silver and carbon pulses was varied in order to obtain samples with various silver contents. Figure 2 shows the contact angle of all the deposited samples. The specimens deposited using ratios of 1 pulse of silver for 12 pulses of carbon or less had overall higher surface energies (i.e. lower contact angles). The lowest contact angle was obtained by the film deposited using 1 pulse of
silver for 12 of carbon. Samples containing higher silver contents had a hydrophilic/hydrophobic behavior with contact angles slightly below 100 degrees. These samples had very similar surface energies which did not change even in the case of the pure silver coating, indicating the high presence of the metal on the surface of samples deposited with 6:1 and 3:1 pulse ratios.

3.2 Surface biocidal effect
As reported in the experimental section, a BacLight live/dead assay was used in order to determine the biocidal effect on the surface of the films prepared with increasing contents of silver. The principle of this assay relies upon the differential staining properties of live bacteria versus dead bacteria. All cells were observed to stain with the cell permeable Sytox-9 fluorochrome dye, while cells simultaneously staining with the cell-impermeable DNA intercalator propidium iodide fluorescence were interpreted to be dead (tainted in red). As can be observed in the microscope fluorescence images of figure 3, there appeared to be a dose dependent decrease in both the total number of adherent cells as well as the percentage of viable cells adherent to the a-C:Ag coated discs. The number of dead bacteria on the surface is higher than the number of live bacteria in figures 3(d)-(f). These were also the samples with higher contact angles and higher concentration of silver.

3.3 Antibacterial efficacy on the medium
Microorganisms can colonize the surfaces of medical devices and form matrixes of bacteria and proteins called biofilms. In order to determine the possible biofilm inhibition effect of the prepared a-C:Ag films on the medium, a fluorometric assay was used to quantify the percent viability of bacteria in suspended medium (Fig. 4). There was an inverse association (r²=0.980) between bacterial viability in suspension and the ratio of silver pulses used to coat the discs, however, statistically significant reductions in the percent viability with respect to the a-C discs of cells in suspension were evident at the highest concentration of Ag (p<0.0001). Similarly, when the suspended medium was transferred to solid media for colony counting, cultures derived from the pure Ag sample (0.28±0.39 CFU/μl) showed a significant reduction in colony forming units relative to the pure a-C coating (43.14±11.78 CFU/μl, p<0.0001), as shown in Fig. 5. However, there was no statistically significant reduction of colony forming units in mixed a-C:Ag films in comparison with pure a-C film.
**Figure 3.** Fluorescent microscopy of *Staphylococcus aureus* adherent to a-C:Ag coated TiAlV discs at ratios of (a) a-C (b) 24:1, (c) 12:1, (d) 6:1, (e) 3:1, and (f) pure Ag. Monochromatic digital micrographs were individually captured with a 100x oil-immersion objective under epifluorescent illumination at either Ex.480nm/Em.535nm (green) to visualize viable cells, or Ex.510nm /Em590nm (red) to visualize dead cells and corresponding images were overlayed. Scale bar = 10μm.

![Fluorescent microscopy images](image)

**Figure 4.** Fluorometric assay of suspended *S. aureus* viability after 24 hours of culture versus a-C:Ag coated discs with varying Ag pulse ratios. Mean of n=4 replicate experiments; bars show 1 standard deviation (SD).

![Fluorometric assay graph](image)

Fluorometric Assay

$y = -46.0x + 65.5$

$R^2 = 0.980$

$*p<0.001$
Figure 5. *Staphylococcus aureus* colony forming units in suspension after 24 hours of culture with a-C:Ag coated discs with varying pulse ratios. Mean of n=2 replicate experiments; bars show 1 standard deviation (SD).

4. Conclusions and future work
In this study, the antimicrobial property of hydrogen-free a-C:Ag films, deposited by the pulsed dual-cathode filtered cathodic arc technique, was analyzed in both the surface of the prepared thin films and in the medium. Microscopically, there was a clear correlation between the number of viable *Staphylococcus* bacteria adhered to the surface and the decreased wettability of the films due to the increased presence of silver in the surface. However, even when there was a drop in the amount of viable cells in the medium in some mixed a-C:Ag films, there was only statistically significant reduction in the number of colony forming units in the medium for the case of the pure silver film. In view of these reported antibacterial results as well as the results reported in other studies on DLC-Ag films, future efforts on the development of silver containing amorphous carbons should focus on studies of biofilm formation as well as in-vivo tests.

5. Acknowledgements
The authors thank J. C. Sánchez-López (ICMSE, CSIC) for his helpful comments. This work was partially supported by MICINN project Consolider (CSD 2008-0023 FUNCOAT). AA acknowledges support by the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

6. References
[1] Endrino J L, Escobar Galindo R, Zhang H S, Allen M, Gago R, Espinosa A and Anders A 2008 Structure and properties of silver-containing a-C(H) films deposited by plasma immersion ion implantation *Surface and Coatings Technology* **202** 3675-82
[2] Hauert R 2003 A review of modified DLC coatings for biological applications *Diamond and Related Materials* **12** 583-9
[3] Hauert R, Gampp R, Muller U, Schroeder A, Blum J, Mayer J, Birchler F and Wintertanet E 1997 Surface analysis and bioreactions on silver-containing amorphous hydrogenated carbon
films American Chemical Society, Polymer Preprints, Division of Polymer Chemistry 38 994-5

[4] Ishihara M, Kosaka T, Nakamura T, Tsugawa K, Hasegawa M, Kokai F and Koga Y 2006 Antibacterial activity of fluorine incorporated DLC films Diamond and Related Materials 15 1011-4

[5] Katsikogianni M, Spiliopoulou I, Dowling D and Missirlis Y F 2004 Bacterial (S. Epidermidis) adhesion on P VC and D LC/Ag/CF$_4$ coatings. In: Transactions - 7th World Biomaterials Congress, (Sydney) p 1082

[6] Katsikogianni M G, Syndrevelis C S, Amanatides E K, Mataras D S and Missirlis Y F 2007 Plasma treated and a-C:H coated PET performance in inhibiting bacterial adhesion Plasma Processes and Polymers 4

[7] Kwok S C H, Zhang W, W an G, McKenzie D R, B ilek M M a nd C hu P K 2007 Hemocompatibility and anti-bacterial properties of silver doped diamond-like carbon prepared by pulsed filtered cathodic vacuum arc deposition Diamond and Related Materials 16 1353-60

[8] Morrison M L, Buchanan R A, Liaw P K, Berry C J, Brigmon R L, Riester L, Abernathy H, Jin C and Narayan R J 2006 Electrochemical and antimicrobial properties of diamondlike carbon-metal composite films Diamond and Related Materials 15 138-46

[9] Narayan R J, Abernathy H, Riester L, Berry C J and Brigmon R 2005 Antimicrobial properties of diamond-like carbon-silver-platinum nanocomposite thin films Journal of Materials Engineering and Performance 14 435-40

[10] Zhang H S, Endrino J L and Anders A 2009 Modification of surface and tribological properties of DLC films by adding silver content. In: 2008 Proceedings of the STLE/ASME International Joint Tribology Conference, IJTC 2008, pp 537-9

[11] Anders A, Brown I G, McGill R A, and Dickinson M R 1998 "Triggerless" triggering of vacuum arcs," J. Phys. D: Appl. Phys. 31, 584-587

[12] Anders A., Pasaja N., and S. Sansongsiri S Filtered cathodic arc deposition with ion-species-selective bias, Rev. Sci. Instrum. 78, 063901-1-5