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# Fabrication of antimicrobial silver-doped carbon structures by combinatorial pulsed laser deposition

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ABSTRACT

We report on the selection by combinatorial pulsed laser deposition of Silver-doped Carbon structures with reliable physical-chemical characteristics and high efficiency against microbial biofilms. The investigation of the films was performed by scanning electron microscopy, high resolution atomic force microscopy, energy dispersive X-Ray Spectroscopy, X-ray diffraction, Raman spectroscopy, bonding strength "pull-out" tests, and surface energy measurements. *In vitro* biological assays were carried out using a large spectrum of bacterial and fungal strains, *i.e.*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Candida albicans*. The biocompatibility of the films obtained was evaluated on MG63 mammalian cell cultures. The optimal combination with reasonable physical-chemical properties, efficient protection against microbial colonization and beneficial effects on human cells was found for Silver-doped Carbon films containing 2 to 7 at.% silver. These mixtures can be used to fabricate safe and efficient coatings of metallic implants, with the goal to decrease the risk of implant associated biofilm infections which are difficult to treat and often responsible for implant failure.



## 1. Introduction

Diamond-like carbon (DLC) can be considered a metastable phase of amorphous carbon (C) that contains, besides hydrogen and $sp^2$ bonded (graphite-like) C nanoclusters, a high fraction of $sp^3$ (diamond-like) sites (Eason, 2007; Ferrari and Robertson, 2004; Robertson, 2008; Popescu et al., 2015). The improvement of DLC films' properties (such as tribological behavior, residual stress state, adherence, corrosion resistance, electrical resistivity and biological response) can be achieved by incorporation of different metal and nonmetal atoms (*e.g.*, Ti, Mo, Cr, W, Au, Al, N, Si and Ag) (Manninen et al., 2015; Wu et al., 2013; Yaremchuk et al., 2015; Liu et al., 2016; Wang et al., 2006; Dwivedi et al., 2013; Choi et al., 2008a; Sedlackova et al., 2005). Moreover, the formation of $sp^3$-C hybridization state by introducing metal phase in amorphous carbon matrix has the role to improve the compressive stress (Yu et al., 2013). Furthermore, in the case of biomedical applications, the next generation of implant-type coatings aims to fulfill two major requirements (*i.e.*, high biocompatibility and resistance to microbial colonization) which can be reached by suitable doping of DLC.

Despite the huge positive impact of antibiotics in the treatment of infectious diseases, their unjustified and massive use led to the emergence of high antimicrobial resistance rates, which are

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responsible for treatment failure and complex health conditions. This phenomenon is caused by the high spreading potential of resistant microorganisms and genes, facilitated by some social factors (*e.g.*, globalization, increased international mobility), increased rate of nosocomial infections and the excessive use of broad-spectrum antibiotics.

Moreover, antibiotic resistance is enhanced by the ability of microorganisms to develop mono- or poly-microbial biofilms on natural tissues (such as skin, mucosal epithelium, teeth) or artificial prosthetic devices (*i.e.*, catheters, dental materials, heart valves, intrauterine devices, joint prostheses) (Lazar and Chifiriuc, 2010a). A bacterial biofilm can be considered a primitive form of differentiation and organization, a proto-tissue equipped with a circulatory system, homeostasis and integrity, similar to the higher organism tissues. Biofilms are considered adaptive structures (Costerton, 2007), which could efficiently disseminate and initiate secondary infections in different sites of the host organism. Biofilm embedded cells show an altered phenotype characterized by reduced growth rates and a different gene expression, which is responsible for their high resistance to antibiotics and other antimicrobial and stress factors (Lazar and Chifiriuc, 2010b; Costerton, 1989; Carpentier, 1999; Donlan, 2002; Grumezescu, 2013). This phenotypic behavioral resistance, described as tolerance (Lazar, 2003), is mediated by multiple molecular mechanisms not fully understood yet, such as: (i) low penetration or impermeability to antimicrobial compounds in the biofilm matrix; (ii) nutritional limitations of biofilm cells into the depth of the biofilm; (iii) presence of functional variants named "persistent" cells; (iv) activation of stress response genes and the development of more tolerant phenotypes (Castrillón Rivera and Palma Ramos, 2012).

Biofilms growing on implants and medical devices have a substantial impact on human health, leading to infections associated with high morbidity and mortality rates. It was shown that 80% of human infections are due to biofilms and are characterized by mild symptoms, chronic course and high resistance to antibiotic treatment (Lazar, 2011). The treatment of biofilm-associated infections usually requires complex therapeutic strategies including a combination of several antibiotics, which often cause high toxicity (Limban et al., 2013; Boucher et al., 2009). The systemic therapies used in the treatment of device-related infections are often ineffective, due to impaired blood circulation and high concentration of antibiotics. The microbial agents involved in severe biofilm-related infections are Gram-positive (*e.g.*, *Staphylococcus epidermidis* and *S. aureus*) and Gram-negative (*e.g.*, *Pseudomonas aeruginosa*) bacteria and fungi (especially *Candida albicans* and *C. parapsilosis*). In multi-specific biofilms, an indirect pathogenicity mechanism occurs, leading to treatment failure: virulent microorganisms with low intrinsic antibiotic resistance are protected from the activity of antimicrobial drugs by antibiotic resistant commensal strains. For example, *S. aureus* and *C. albicans* coexistence in a biofilm amplifies the *S. aureus* resistance to vancomycin (Jenkinson and Lamont, 2005).

The increasing incidence of implant and medical devices associated infections requires the development of new strategies to delay or prevent the adhesion of microbial cells to synthetic material surfaces and the formation of microbial biofilms. Once mature and protected by the extracellular, self-secreted matrix, biofilms become very resistant to antimicrobial agents (Vasilev et al., 2009).

One of the preferential research directions relies on obtaining nanostructures, containing highly-efficient antimicrobial agents. They are able to prevent or reduce microbial adhesion and biofilm development, while providing excellent biocompatibility for human cells (Simchi et al., 2011).

Silver (Ag) is recognized for its antimicrobial broad-spectrum activity against Gram-positive and Gram-negative bacteria, including multidrug-resistant microbial strains, fungi and protozoa, as well as for its anti-viral properties (Monteiro et al., 2009; Paneva et al., 2011; Pop et al., 2015; Endrino et al., 2010).

Consequently, Ag-incorporated DLC (Ag:DLC) thin films have recently gained significant attention in the realm of biomedical applications. Several deposition techniques have been proposed for their fabrication: Direct Current (Onoprienko and Danylenko, 2012; Manninen et al., 2013) and Radio Frequency Magnetron Sputtering (Dhandapani et al., 2014), Chemical Vapor Deposition (Dwivedi et al., 2013; Marciano et al., 2009; Paul et al., 2010), Pulsed Filtered Cathode Vacuum Arc (Kwok et al., 2007), Multi-Ion Beam Assisted Deposition (Yu et al., 2013), Ion Implantation (Batory et al., 2014), and Pulsed Laser Deposition (PLD) (Morrison et al., 2006; Narayan, 2005).

The attractiveness of Ag:DLC as coatings for biomedical implants also originates in their excellent corrosion and wear resistance (Dhandapani et al., 2014; Baba et al., 2013), antibacterial properties (Narayan, 2005; Baba et al., 2013) and hemocompatibility (Choi et al., 2008b). However, the doping interval, which provides the synthesized structures with antimicrobial properties, can vary slightly and it was suggested that this depends on the deposition method (Onoprienko and Danylenko, 2012; Manninen et al., 2013; Dhandapani et al., 2014; Morrison et al., 2006; Bociaga et al., 2015).

The role of Ag incorporation in carbonaceous structures is still under debate. A silver content of up to ~13 at.% increases the hardness of DLC layers and improves their adherence to the substrate (Manninen et al., 2013; Wang et al., 2009) and tribological behavior (Dhandapani et al., 2014; Wang et al., 2012). Above this threshold, the hardness values decrease considerably (Wang et al., 2009). However, other studies reported a reduction of the hardness when Ag is present in DLC structures (Wu et al., 2013; Dhandapani et al., 2014).

Another remarkable advantage of DLC coatings arises from their renowned resistance to structural, chemical and morphological modification that can be inflicted by the various sterilization procedures (*e.g.*, even repeated steam or chemical autoclaving cycles) required prior to implantation. Furthermore, DLC coatings are known to withstand the harsh conditions of the sour autoclave per NACE TM0185 testing standard (Anon., 2016) or saline high temperature and high pressure environments (Wang et al., 2014).

An advanced extension of PLD, the Combinatorial PLD (c-PLD) method was proposed for the fabrication of thin films as it elicits great flexibility and the ability to generate concentration gradient "libraries" along the surface of a given substrate by the simultaneous ablation of two or more targets (Eason, 2007; Hata et al., 2007; Craciun et al., 2009; Socol et al., 2011; Eason et al., 2014). The c-PLD stands for a cost-efficient technique with real capabilities to shorten the path toward mass fabrication.

The main objective of this study was to investigate the bonding strength, antimicrobial activity and biocompatibility of Ag-doped C (C:Ag) films deposited on titanium (Ti) implants. c-PLD was used to determine the appropriate Ag doping levels for laser synthesized DLC films, and to develop reliable designs of a new generation of multi-functional coated implants with increased resistance to microbial colonization and high biocompatibility.

## 2. Material and methods

### 2.1. Combinatorial pulsed laser deposition experiment

In search for the optimal coatings able to elicit the desired mechanical and biological properties, the compositional and structural C to Ag libraries were deposited by the c-PLD technique.

All experiments were carried out in a stainless steel reaction chamber. The samples were obtained by c-PLD method using high