Cold atmospheric plasma assisted deposition of nanostructured coatings to reduce biofilm adhesion and proliferation

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Abstract: In this work, a cold atmospheric plasma assisted process to produce anti-biofilm and anti-clot nanostructured coatings composed by silver nanoparticles embedded in a plasma polymerized matrix is presented. Chemo-morphological and biological characterizations of multilayer films deposited both on 2D and 3D substrates outline coating potentialities against biofilm adhesion and clots formation. Coatings stability upon liquid immersion and ethylene oxide sterilization are promising features towards future *in-vivo* tests.
Keywords: in-flight synthesis, antibacterial coating, silver nanoparticles, plasma jet.

1. Introduction

In the last decades, the development of innovative strategies for the design and production of devices to be used in biomedical applications has raised a significant interest. Enormous efforts are devoted to the development of new medical devices (MDs) having functional coatings able to increase specific properties (bioresorbability, biocompatibility, mechanical and chemical properties, cell selectivity, etc) while preserving bulk mechanical performances [1]. In particular, antibacterial functionalities are raising a significant interest worldwide since bacterial colonization of MDs is one of the main sources of nosocomial hospital-acquired infections, often related to multi-drug resistance bacteria contamination and biofilm formation. Among MDs subjected to bacterial contamination, central venous catheters (CVCs) are often related to bloodstream infections that increase morbidity and mortality, causing one third of hospital related bacterial contaminations with a 15-20% of mortality [2]. In many cases, central venous catheter related bloodstream infections (CRBSIs) can be ascribed to MD contamination from patient's skin micro-organism, that, through bacteria growth and proliferation, could lead to biofilm formation on the surface of CVCs. In this perspective, the reduction of bacterial adhesion/proliferation is the first and fundamental step towards the decrease of biofilm formation and the incidence of CRBSIs. CVCs, widely employed in critically-ill patients for the administration of drugs and nutritional solutions, are also affected by platelets adhesion and clots formation, strongly increasing the risk of venous thromboembolism and promoting bacterial adhesion and biofilm proliferation [3]. In this perspective, the control of clots formation and the reduction of biofilm proliferation on CVCs' surfaces by means of surface functionalization appears as an important preventive measure against bloodstream infections.

In the last years, different approaches have been reported for the development of antibacterial and anticlot functionalities through surface modification: bacterial adhesion and clots formation could be avoided by means of non-fouling surfaces, while cell death could be reached introducing antimicrobic compounds on MDs.

Among the innovative technologies employed for the surface modification of biomaterials, plasma functionalization and coating deposition have raised significant interest for being solvent-free and for operating at low temperatures, thus enabling the treatment of thermosensitive materials. Thanks to its versatility in terms of the plasma sources and the precursors that can be employed, atmospheric pressure cold plasmas can be used in the deposition of silver containing coatings able to reduce bacterial growth [4]. Even if a significative number of scientific works deals with plasma assisted deposition of silver containing antibacterial coatings, few of them face the problems related to process scale-up and to in-vivo application of MDs. Beier et al. [5] for example have reported on the antibacterial properties of plasma deposited AgNPs based coating, but they have not investigated health risks related to NPs dispersions in biological environment. In order to prevent AgNPs leakage and control silver ions release, Deng et al. [6] have developed a double layer plasma deposited silica-like coating: after immersion in an AgNPs containing solution, NPs were fixed on a first plasma deposited coating by means of a thin barrier layer. Deposited coatings exhibited antibacterial promising properties against different types of pathogens usually responsible of fungal infections. The antibacterial properties of plasma produced coatings were preserved also after several type of washing cycle, highlighting interesting perspectives for the application of plasma coating tecniques in the biomedical field.

In this framework, the aim of this work is to develop and characterize a cold atmospheric plasma assisted process for the deposition of nanostructured coatings characterized by antibiofilm and anti-clot properties on polymeric substrates to be employ for the production of CVCs. Deposited multilayer coatings were composed by AgNPs, directly synthesized in the plasma region through the reduction of a silver salt contained in an aerosol and embedded in a plasma polymerized HMDSO (ppHMDSO) matrix. Silver containing coating deposited both on 2D substrate and 3D mini catheters were characterized using chemo-morphological tecniques. Biocompatibility and biological assays were carried out according to ISO normative, as a first step towards animal *in-vivo* tests.

2. Materials & Methods

Nanostructured coatings were deposited by means of a single electrode corona plasma jet driven by an AlmaPulse (AlmaPlasma srl) high voltage pulse generator working at 10 kV and 14 kHz. The plasma source is equipped with two different gas injection channels: a primary gas sustains the plasma discharge and flows around the electrode while a secondary gas is introduced directly in the plasma plume through a gas diffuser (Figure 1). A multistep approach was used to deposit coatings composed of three distinct layers on $BaSO_4$ (used as a radiopaque compound) loaded polyurethane 2D substrates ($30x30x3mm^3$): a first buffer layer composed of ppHMDSO; a second layer composed of AgNPs directly synthetized in the plasma region through plasma assisted reduction of $AgNO_3$ aerosol droplets; a third thin layer of ppHMDSO that acts as a barrier layer preventing the dispersion of AgNPs in the biological environment and increasing coating biocompatibility. The three deposition/synthesis steps lasted for 60, 300 and 30 seconds, respectively. The deposition of the buffer and barrier layers was carried out by injecting in the primary channel 1.7 slpm of argon (Ar) carrying 0.2 g/h of HMDSO; 2.4 slpm of Ar were introduced in the secondary channel to increase the volume of the plasma plume and, consequently, the treatment area. The in-flight synthesis of AgNPs was performed introducing in the primary channel 1.7 slpm of Ar and in the secondary channel an aerosol of 250 mM $AgNO_3$ solution carried by 2.4 slpm of Ar.

The chemical characteristics of the coatings were analysed by means of Fourier Transform Infrared spectroscopy in ATR mode (ATR-FTIR, Agilent Cary 660), while substrates morphological characteristics were investigated using a Scanning Electron Microscopy and Energy Dispersive X-ray microanalysis (SEM-EDX, Phenom G2 ProX) on preliminary gold palladium sputter coated samples (SC7620 Mini Sputter Coater, Quorum Technologies). With the aim to evaluate the biocompatibility of deposited coatings, hemocompatibility tests were performed by means of dynamic human blood contact: treated substrates were dipped in human blood and incubated at 37°C for 24h using an orbital shaker. Hemolysis was evaluated by haemoglobin free assay (Hemoglobin Assay Kit Sigma MAK115) according to ISO10993-4 [5]. After blood contact, biomaterials were stained with haematoxylin/eosin: substrates were fixed in formalin (10%v/v, 15 minutes), stained with haematoxylin, washed with tap water, counterstained with eosin (20 seconds) and washed in double distilled water (ddH2O). Antibiofilm performances were evaluated by soaking the treated substrates in a four strains broth bacterial culture (Bacillus subtillis, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus hirae 1:1:1:1, 10^8 CFU/ml) for 10 days at 37°C. Crystal violet (CV) staining was used to estimate biofilm adhesion and proliferation on the coated biomaterials: substrates were fixed in methanol (-20°C) for 2 minutes, washed in ddH20 and dipped in crystal violet (0.4%) for 5 minutes [6]. After staining procedures of the substrates, clots and biofilm formation was evaluated by means of stereo microscope imaging (Microscope for Large Fields AxioZoom V16, Carl Zeiss Microscopy GmbH).

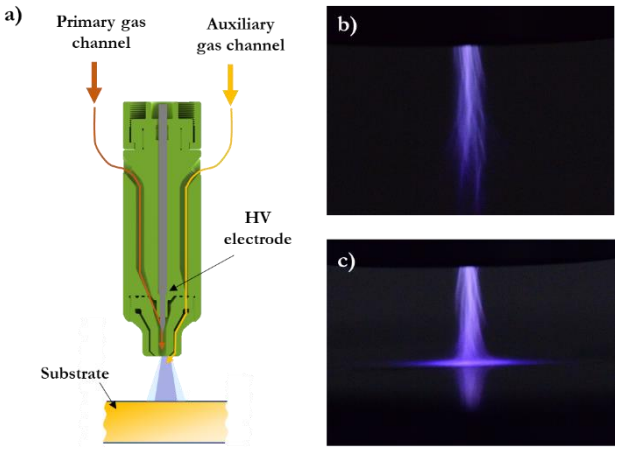

Fig. 1. Single electrode corona plasma jet: a) plasma source schematic; b) free flow plasma discharge; c) plasma discharge on a dielectric substrate

Afterwards, the developed multistep process was employed for the deposition of nanostructured coatings on the external surface of mini catheters (50 mm length, 0.8 mm OD, 0.4 mm ID). After coating deposition, the mini catheters were sterilized by means ethylene oxide (EtO) and SEM-EDX analysis were performed in order to investigate coating stability. In-vitro biocompatibility of plasma treated mini catheters was assessed my means MTT test according to ISO 10993-5 [7]: after biomaterial elution in cell culture medium for 24h at 37°C, the eluate was added to L292 culture and cells viability was assessed by colorimetric evaluation through MTT reduction. As suggested by ISO, latex and high-density polyethylene (HDPE) were used respectively as positive and negative controls.

3. Results

The chemo-morphological characterization of deposited coatings was performed by means of ATR-FTIR and SEM-EDX. The obtained IR spectra, reported in Figure 2, outline

the deposition of organosilicon coatings on all treated substrates: plasma coated substrates exhibit a strong absorption at 1070 cm^{-1} that can be ascribed to Si-O-Si asymmetric stretching. The lower absorption bands at 441 cm-1 and 900 cm^{-1} could be assigned respectively to rocking vibration and bending of Si-O-Si bonds, while the broad peak centred at 3300 cm^{-1} is due to stretching of OH groups probably generated from the reaction of monomer fragments during deposition. The hydrophobic behaviour of the deposited film, which displays a contact angle of 91°, highlights its organic character: according to the scientific literature, these results confirm the PDMS-like nature of the coating, consistent with a deposition process carried out by means of an oxygen-free plasma discharge. Finally, stability tests upon 72h of water contact confirm that deposited coatings are water stable and therefore suitable for biomedical application.

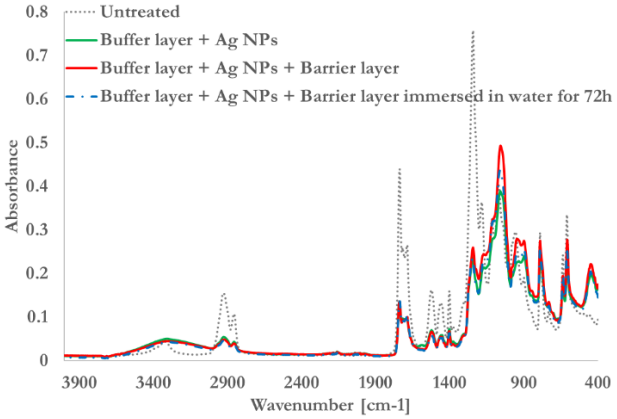

Fig. 2. ATR-FTIR spectra of the deposited coatings

SEM-EDX analysis performed on treated substrates confirm the deposition of nanostructured coating, in which AgNPs are embedded. With respect to the untreated substrate, as reported in figure 3, ppHMDSO coating displays a smoother morphology while $BaSO_4$ particles covered by coating are still detectable. When AgNPs directly synthesized in the plasma region were deposited on ppHMDSO buffer layer, NPs aggregates are visible on the surface of treated material and the presence of Ag is confirmed by EDX analysis. The barrier layer (figure 3d) incorporates the AgNPs preventing their dispersion: SEM analysis shows the presence of small cracks on coating surface while EDX spectra highlights a reduction of Ag concentration, as a consequence of the embedding process.

Haemoglobin free assay carried out on blood plasma highlights that the deposited coatings does not significantly affect hemolysis rate and biocompatibility of the pristine substrate (with respect internal control, data not shown). Haematoxylin/eosin staining of untreated and ppHMDSO coated substrates after dynamic contact with blood shows the presence of fibrine/plasma deposits on the biomaterials surface: obtained results show that a large number of deposits (probably clots) can be detected. Moreover, magnified images of untreated samples and ppHMDSO coated substrates (Figure 4B, 4F) highlight the presence of fibrine and plasma deposits which are attached to the biomaterials surface. When AgNPs are embedded in ppHMDSO, clots formation was strongly reduced and no fibrine/plasma deposit is observed (Figure 4C, 4D). The greatly improved anti-clot properties of nanostructured coatings can be ascribed to AgNPs antiplatelet properties. As far as antibiofilm performances is concerned, microscope analysis carried out at different magnification highlight the formation of biofilm structures on untreated substrates: bacterial colonies of big dimension (more than 600 um) can be found both at the edge and in the middle of the sample. Substrates coated with ppHMDSO exhibit a strongly reduced biofilm adhesion with respect to untreated substrates. When AgNPs are added to ppHMDSO coating, cells adhesion to biomaterial is further reduced with respect to ppHMDSO coated substrates: higher magnification images highlight the presence of only small regions of biofilm adhesion at the edge of coated sample. We can conclude that the presence of a deposit of ppHMDSO in which NPs are embedded can strongly reduce biofilm adhesion and proliferation with respect to untreated substrate. The anti-biofilm properties of deposited coatings can be referred to the presence of AgNPs whose antibacterial properties are widely known; moreover, according to SEM analysis, substrates roughness was strongly reduced after coatings deposition, potentially reducing biofilm adhesion.

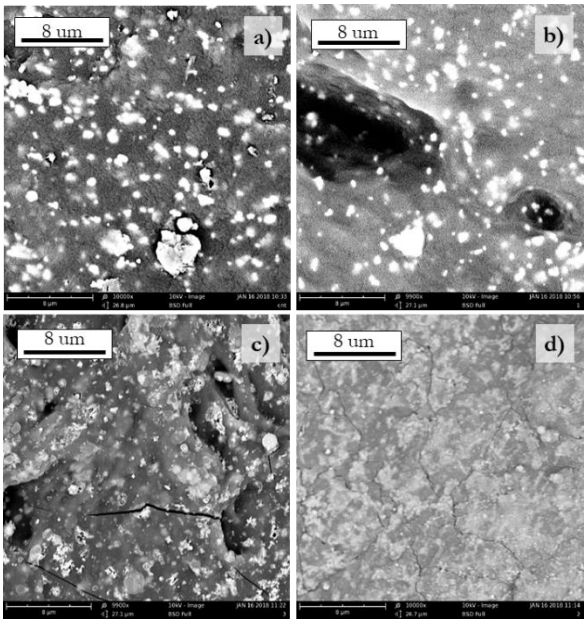

Fig. 3. SEM analysis of the deposited coatings: a) untreated; b) substrate coated with ppHDSO; c) substrate coated with ppHDSO and AgNPs; d) substrate coated with ppHDSO, AgNPs embedded in ppHMDSO barrier layer

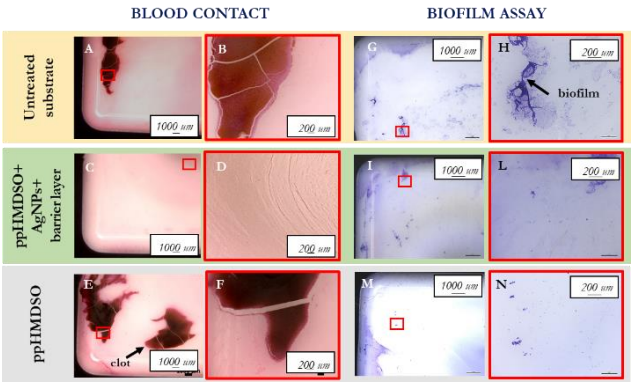

Fig. 4. Results from biological assays on treated substrates

Starting from the promising biological and chemo-morphological results obtained on 2D substrates, the multistep deposition process was applied to mini catheters. SEM-EDX analysis highlights the presence of the coating on the whole length of plasma treated mini catheters. In order to investigate the stability of the deposited coating, SEM-EDX analysis performed on EtO sterilized biomaterials confirmed the results obtained on 2D substrates in terms of stability. Slight variations of the coating morphology can be observed after EtO sterilization and after 72h of immersion in broth: the acquired images, reported in figure 5, outline that after immersion in TSB the surface of the coating is smoother and more uniform with respect to the one only subject to EtO treatment.

The biocompatibility of plasma treated mini catheters evaluated with MTT test is confirmed in all sample analysed (untreated substrate, ppHMDSO coated substrate, ppHMDSO coated with AgNPs embedded in ppHMDSO matrix substrate). Cell viability for treated material is over the 70%, the minimum viability level required by ISO standards, pointing out that developed material could be employed for in-vivo tests (data not shown).

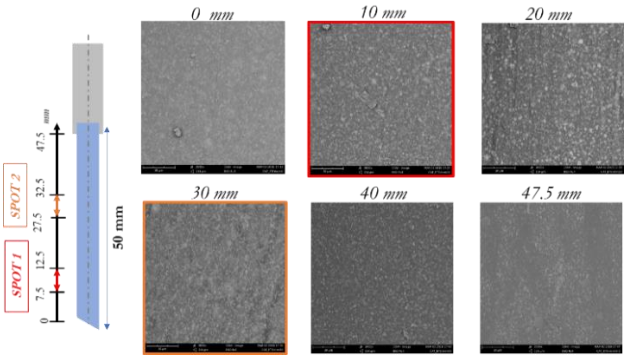

Fig. 4. SEM analysis of deposited mini catheter after EtO sterilization and TSB immersion

4. Conclusion

The obtained results outline the potentiality of the use of a cold atmospheric pressure plasma assisted process for the deposition of multilayer coatings with embedded AgNPs. A multistep process has been developed and experimentally studied: plasma assisted polymerization was employed to deposit thin organosilicon films on 2D and 3D substrates, while the reactive species generated in the plasma discharge were instrumental for the in-flight reduction of silver ions and the synthesis of AgNPs through aerosol treatment. The deposited coatings exhibit great stability upon liquid immersion also after EtO sterilization. Stability upon sterilization and liquid contact is an important requirement for biomaterials: on one hand, sterile MDs are required in order to prevent cross-contamination during their application (in particular for CVCs), while on the other hand the release of materials in biological environment has to be avoided. According to biological assay results, innovative biomaterials on which nanostructured coatings were deposited can reduce biofilm adhesion and clot formation with respect to untreated substrates. These results appear to be an important first step towards the development of an innovative process to be used for the surface modification of biomaterials and future biological in-vitro and in-vivo tests. Further efforts need to be devoted to the optimization of the synthesis and deposition processes in order to control the coating characteristics and functionalities.

5. References

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