Journal of Medical Microbiology and Infectious Diseases

JoMMID
ISSN: 2345-5349
eISSN: 2345-5330

Bioactivity Determination of Recombinant lysostaphin Immobilized on Glass Surfaces Modified by Cold Atmospheric Plasma on *Staphylococcus aureus*

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ARTICLE INFO

Original Article

Keywords: Recombinant Proteins, Lysostaphin, Cold Atmospheric Plasma

Received: Nov. 11, 2019
Received in revised form: Dec. 15, 2019
Accepted: Dec. 16, 2019
DOI: 10.29252/JoMMID.7.4.132

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ABSTRACT

Introduction: *Staphylococcus aureus* is a source of nosocomial infections and one of the significant concerns in patients with indwelling devices. Lysostaphin is a bacterially produced endopeptidase with a unique activity on *S. aureus*. Plasma, the fourth state of the material, consists of charged ions, free electrons, and activated neutral species. Biomedical applications of cold plasma are rapidly growing due to its capacity to treat heat-sensitive objects such as polymeric materials and biological samples. It activates surfaces by etching them to stabilize proteins. The direct effect of cold atmospheric plasma on the eradication of microorganisms have been investigated. However, there is no report on immobilizing antibiotic agents. **Methods:** In this study, the lysostaphin protein was expressed and purified using Ni-NTA column, then the purified enzyme was immobilized on glass surfaces pretreated with cold atmospheric plasma for 150 s, 200 s, and 300 s. The antimicrobial activity of immobilized lysostaphin on *S. aureus* was approved by *in vitro* analysis. **Results:** The 300 s plasma treatment confirmed to be the best time arrangement for more lysostaphin immobilization, shown by Atomic Force Microscopy. **Conclusion:** Our results showed that passive adsorption to the treated surface does not affect the structure and subsequent antimicrobial function of the recombinant protein compared to the standard proteins.

INTRODUCTION

The emergence of "super bacteria" resistant to antibiotics, such as methicillin-resistant *Staphylococcus aureus*, calls for novel antimicrobial therapies to prevent extended hospitalizations and deaths [1].

Worldwide, about 2 billion people are estimated to carry *S. aureus*, of which up to 53 million are infected with methicillin-resistant *S. aureus* (MRSA) [2]. Lysostaphin is a cell lytic enzyme with specific bactericidal activity against *S. aureus* while saving the bacterial flora where it is administered [3]. The use of lysostaphin to generate an antistaphylococcal agent stabilized onto the surfaces is an important task to control/prevent the infection. Miao *et al.* (2011) employed a cell lytic enzyme, lysostaphin, with specific bactericidal activity against *S. aureus*, to generate anti-infective bandages [1]. In line with this study, Shah *et al.* (2004) demonstrated significant antimicrobial activity against *S. aureus* by physically adsorbed lysostaphin on intravenous catheters with high *in vitro* efficacy [4].

Most of the polymers, commonly used for biomedical applications, need modifications in surface properties by chemical or physical tools, *e.g.*, plasma. Plasma is the state of ionized gas that, based on the thermodynamic equilibrium of energy of its particles, is defined as hot or cold [5]. The latter is applied for the treatment of heat-sensitive objects such as polymeric materials and biological samples without introducing damages. During the process, gas particles such as ions, electrons, radicals, neutrals, and UV radiation etch the surface in the nanoscale and lead to modified functional properties of the matrix [6].

Recek *et al.* (2013) have used this characteristic of plasma to study protein adhesion and cell response to plasma-treated polymer surfaces, such as absorption of albumin protein on the modified surfaces [7].

One of the effective ways of killing bacteria is the coating of the active surface with the specific antibiotic. Jose *et al.* (2005) showed that vancomycin covalently attached to titanium still bound soluble bacterial peptidoglycan, reduced *S. aureus* colony-forming units by 88% ± 16% over 2 h, and retained antibacterial activity upon a repeated challenge [8].

In this regard, the attachment of penicillin on the chemically modified surface and the antibacterial activity was evaluated by measuring the absorbance percentage of the suspension at 600 nm. The lowest absorbance was

[DOI: 10.29252/JoMMID.7.4.132]

observed for the solution containing penicillin on a modified surface to eradicate *S. aureus* [9].

This study investigates the immobilization of recombinant lysostaphin antibiotic on the glass using cold atmospheric plasma technique followed by evaluation of its antibacterial bioactivity.

MATERIAL AND METHODS

Expression and purification of recombinant lysostaphin. The recombinant pET32a (pET32a-lysostaphin) was generously provided by Dr. Abtahi (Arak Medical Science University) [10], was transformed into *Escherichia coli* BL21 (DE3) as an expression host. A single colony of transformed bacteria was inoculated into a 5 mL nutrient-broth (NB) medium containing ampicillin (100 µg/mL) and incubated at 23℃ and 37℃ for 16 h. Dilutions of 1/100 of bacteria were grown in 50 ml NB until the culture reached an OD600 of 0.6. After recovering 10 ml of the suspension for using as a control, isopropyl-β-D-thiogalactopyranoside (IPTG) with the final concentration 0.5 mM, 2 mM, and 4 mM was added to transformed bacteria and incubated for 4 h. The samples from control bacteria and expressed bacteria were loaded on 12% SDS-PAGE. The recombinant protein contained a polyhistidine (6xHis) sequence. Thus, the Ni-NTA column (Qiagen, Crawley, United Kingdom) was used for purification of lysostaphin under native conditions according to the manufacturer's instruction. Briefly, the pellet was suspended in a binding buffer containing 10 mM imidazole. Then lysozyme was added to the suspension at the final concentration 0.01 mg/ml followed by sonication on ice. Following the addition of 1 µl of DNase I (5 µg/ml), 1 ml of Ni-NTA (50% gel) was added to the collected supernatant and rotated for 1 h at 4℃. After that, the mixture was loaded on an empty column containing a fixed filter. The column was washed four times with washing buffer, and the lysostaphin was eluted with three volumes of an elution buffer containing 200 mM imidazole. The purified protein was dialyzed against PBS at 4℃ overnight, the quality of purified lysostaphin was analyzed on a 12% SDS-PAGE and the purified protein was quantified using the Bradford technique. For further activity analysis, when needed, lysostaphin protein was concentrated using a vacuum dryer apparatus (Eppendorf concentrator 5301, USA).

Disk diffusion antibacterial activity assay of lysostaphin. Sterile 0.5 mm thick filter paper disks (10mm diameter SIGMA 74146-25DISKS-F) were impregnated with 15 µg of purified lysostaphin. The disks were allowed to dry at room temperature at the sterile condition. A single colony of *S. aureus* ATCC 6538 (Microbial Collection of Pasteur Institute of Iran) and *E. coli* top10 strains were incubated in brain-heart infusion broth at 37°C. The overnight bacteria cultures with OD 0.6 were spread on brain-heart infusion agar, and the disks were placed on plates. After 16 h at 37℃, the diameter of zones of inhibition was measured.

Cold atmospheric plasma jet application. The plasma device used in this study was a Dielectric Barrier Discharge (DBD) that consisted of helium gas with a power supply of 10 kV pulses, a frequency of 6 kHz, and an external electrode as the earth [11]. The applied voltage to the electrode ionized the inlet gas fed into the tube, and the generated plasma was emitted out from the end of the tube. From where, it propagated about a 3 cm into the sample using glasses (Hemocytometer MENZEL, Germany) with 0.4 mm thickness and the size of 26 × 20 mm. An area with 0.5 cm × 0.5 cm on glasses was determined and exposed to helium cold atmospheric plasma for 150, 220, and 300 seconds. Then, 15 µg of recombinant and commercial lysostaphin was spotted on the defined area and allowed to dry. Later, the samples were studied with Atomic Force Microscopy (Dual Scope C-26, DME Company) at Institute for Color Science & Technology.

Bioactivity of the immobilized Lysostaphin. Three slides were sterilized by exposure to UV for 20 min on each side. Cold atmospheric plasma discharge was performed for 300 s on the determined area of three slides. Then, 15 µg of recombinant lysostaphin and standard lysostaphin (Sigma) loaded on the cold atmospheric plasma-treated area of two slides. The lysostaphin-contained slides dried and then rinsed three times with 1 ml double distilled water and allowed to dry. The flow-through from the two slides were collected for determination of the amount of un immobilized and shed proteins on each slide using the BCA Protein Quantification Kit (Pars Tous, Iran). A cold atmospheric plasma-treated slide without lysostaphin was considered as the negative control.

A single colony of *S. aureus* was inoculated into 5 ml Luria Bertani (LB) medium and incubated overnight. The OD 600 was measured (~1.8) and it was 2000 folds diluted to 1.5 $\times 10^5$ bacteria/ml. Amounts of 50 µl of *S. aureus* suspensions were loaded on the cold atmospheric plasma-treated area and incubated for 6 h at 37°C in a humid box. Then, each glass located in a 50 ml sterile Falcon tube containing 15 ml LB medium and shaken for 15 min in gentle horizontal rotation. Later, 100 µl of suspension and a 10-times diluted suspension were spread on LB agar and the CFU were calculated. The graphical abstract of the procedures is presented in Fig. 1.

Water contact angle. To measure the wettability of the surface, the static water contact angles (θ) were determined by the drop method at room temperature [12]. A droplet (5 µl) of water was placed on the untreated and 150 s helium plasma-treated on the defined surface area. The water contact angle (θ) was observed using the optical contact angle (OCA) measurement.

RESULTS

The pET32a containing the lysostaphin gene was transformed into BL21. Lysostaphin is a 27 KDa protein, however, the fused proteins, encoded by existing genes in the vector, such as thrombin and Trx-tag has increased the size of the recombinant lysostaphin protein to approximately 46 kDa. The results showed that the best concentration of IPTG and time following induction for higher expression of the recombinant protein were 0.5 mM and 4 h, respectively. The lysostaphin expression at 23°C compared to 37ºC showed a slight increase in the expression of soluble protein in the former one (Fig. 2, lanes 2 and 3).

[DOI: 10.29252/JoMMID.7.4.132]

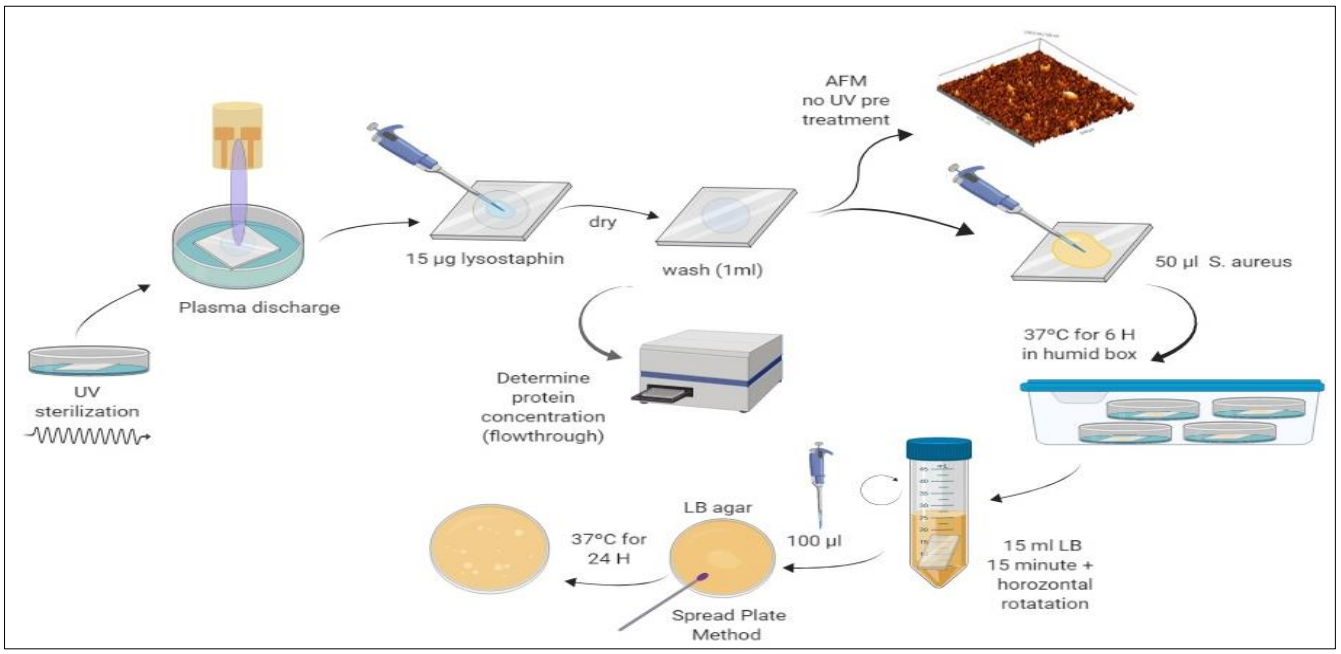

Fig. 1. Comprehensive Scheme of CAP treatment and methods used in this study.

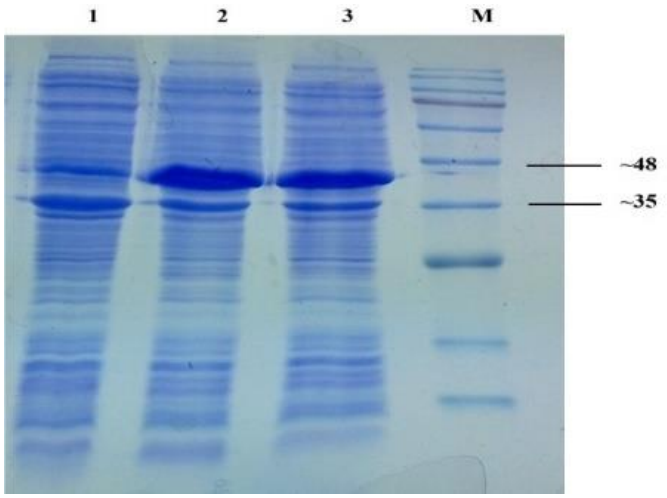

Fig. 2. SDS-PAGE analysis showing the effect of temperature on the expression of the recombinant lysostaphin. Lane 1, uninduced cell extract from *E. coli* BL21(DE3) +pET-lys; lane 2, the supernatant of cell lysate of bacterial culture at 23°C; lane 3, the supernatant of cell lysate of bacterial culture at 37°C

Lysostaphin protein was purified via Ni-NTA agarose. The yield of the purification process was ≈40 µg of purified lysostaphin per milliliter of media. To determine the bioactivity of the purified lysostaphin, zones of inhibition were determined on standard *S. aureus* as a positive control. However, there was no zone of inhibition on *E. coli* that was used as the negative control. The results showed a 16 mm diameter of inhibition zone for 15 µg of purified lysostaphin loaded on the disk for *S. aureus*, with small or no zone of inhibition for *Staphylococcus epidermidis* and *E. coli* strains, respectively.

Analysis of surface modification pattern by Atomic Force Microscopy (AFM). To obtain additional information regarding the surface characteristics of the plasma-treated samples, AFM analyses (Dual Scope C-26, DME Company) were performed to determine the surface topography that can also influence protein adhesion. Our results showed a distinct change in the surface structure following helium plasma treatment after 150 s, 220 s, and 300 s (Fig. 3B, 3C, and 3D), compared to the untreated surface (Fig. 3A). The surface morphology significantly changed with the increased treatment time. For example, a comparison of 150 s (Fig. 3B) 300 s (Fig. 3D) treatment showed more peaks and valleys in the latter and more sharp fragments on the modified surface in the former.

Prolonging the exposure time of cold atmospheric plasma accumulates the modification of surfaces and subsequently the immobilization of lysostaphin. This is shown in Fig. 3E,

[DOI: 10.29252/JoMMID.7.4.132]

F and G for 150, 220, and 300 s treatments, respectively. The results obtained by the AFM technique are restricted mainly to 3D images of the treated samples solution.

The water contact angle is used to measure surface hydrophilicity by evaluating how much a water droplet could spread on a surface. The lower the contact angle, the more hydrophilic the surface becomes. The helium plasma can effectively improve the hydrophilicity of the surface. As a surface becomes more oxidized or has a higher number of ionizable groups, hydrogen bonding with the water molecules becomes easier and the droplet spreads along the hydrophilic surface, resulting in a lower contact angle. Images of water droplets on the sample surface before and after aging on 150 s plasma treatment are presented in Figure 4. The surface treatment by helium non-thermal plasma decreased the 30° water contact angle to 10°.

Antibacterial activity of lysostaphin-immobilized on glasses by cold atmospheric plasma. The amount of immobilized lysostaphin following cold atmospheric plasma treatment of glass surfaces was determined to be 2.5 µg of standard lysostaphin and 10.4 µg of recombinant lysostaphin. Following the addition of *S. aureus* suspension, the negative control (no lysostaphin) showed 7.2×10^8 $CFUml^{-1}$. However, there was no growth of bacteria following 6 h of co-incubation with glasses covered with the same amount of standard and recombinant lysostaphin.

DISCUSSION

Since the introduction of the methicillin for treatment of penicillin-resistant *S. aureus* infections in 1961, there have been reports of isolation of multi-resistant *S. aureus* strains [13], which promoted the search for new antibacterial agents. Lysostaphin is an antibacterial protein from *S. simulans* biovar *Staphylolyticus* that has shown potential in the treatment of *S. aureus* infections via topical and systemic usage [10]. The crucial issue for large-scale application of the enzyme is its availability and low cost of production.

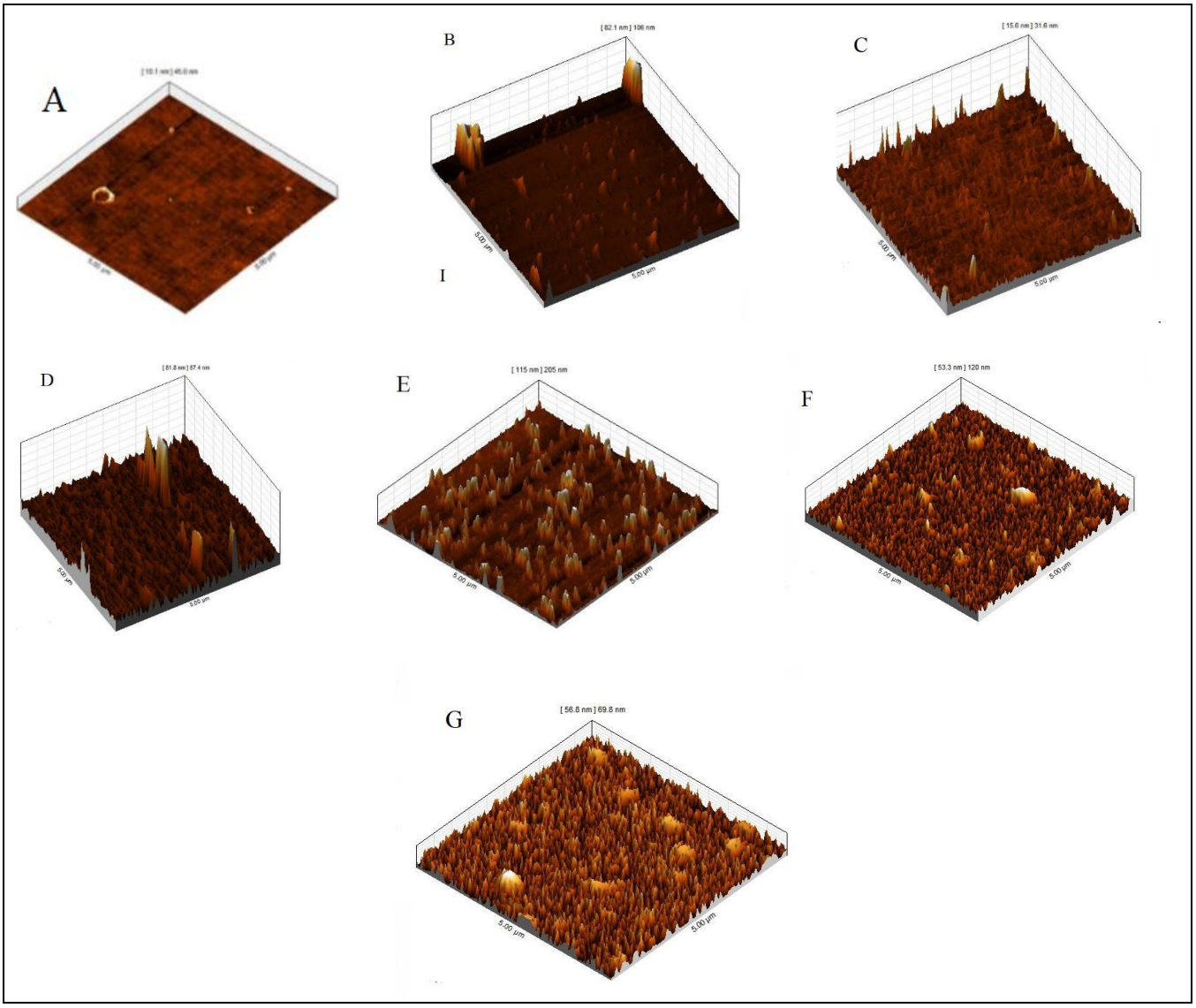

Fig. 3. (A) 3D AFM images of 5µm × 5µm of untreated sample; B) 3D AFM images of 5 µm × 5 µm of surfaces treated with Helium plasma for 150 s (B), 220 s (C) and 300 s (D); 3D AFM images of 5 µm × 5 µm of surfaces treated with Helium plasma covered with immobilized lysostaphin protein for 150 s (E), F (220 s), and 300 s (G)

[DOI: 10.29252/JoMMID.7.4.132]

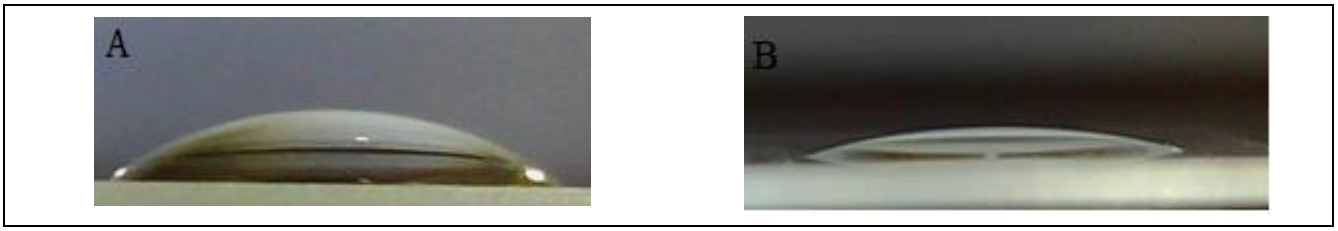

Fig. 4. A water droplet on A) the non-treated surface that produced 30º angle and B) 150 s helium-treated surface that produced a 10° angle

In several studies, recombinant lysostaphin was produced through different vectors, including pET23b+ with a yield of 20 mg/L and pET32a with a yield of 30 mg/L of purified protein of *E. coli* [10, 14]. In the present work, 40 mg/l of recombinant lysostaphin was obtained and the bioactivity of purified protein was tested by the disk diffusion assay method. Kusuma *et al.* (2005) tested 57 different strains of *S. aureus*, which revealed inhibition zones of ≥11 mm using disks carrying 50 µg commercial lysostaphin [3]. However, in the current study, a disk containing 15 µg of recombinant lysostaphin exhibited an inhibition zone of 16 mm in diameter.

One of the abilities of *S. aureus* is to form biofilms on the surfaces, especially on the implantable devices in the health system. Significant attention has been given to the treatment of surface contaminations by minimizing bacterial adhesion and developing bactericidal methods [15].

Furthermore, lysostaphin has shown to be a promising agent for the degradation of biofilm of *Staphylococci* on surfaces [13]. Wu *et al.* (2003) revealed that lysostaphin not only inhibits *S. aureus* growing in biofilms but also disrupts the extracellular matrix of the biofilms *in vitro* on plastic and glass surfaces in 1 µg/mL. Therefore, biofilm formation may be inhibited by immobilizing lysostaphin on the surfaces [16].

The application of plasma techniques for surface modification and subsequent immobilization of biomaterials has become increasingly common [5]. Cold Atmospheric Plasma is non-thermal flow and warms the surface to less than 40°C at the point of application. Cold atmospheric plasma can be produced by several methods such as DBD, Atmospheric Pressure Plasma Jet (APPJ), plasma needle, and plasma pencil [17]. DBD that is used in this study has many applications including sterilization of living tissue, bacteria inactivation, angiogenesis, surface treatment, and excimer formation. Various gases can be used to produce cold atmospheric plasma such as Helium, Argon, Nitrogen, Heliox (a mix of helium and oxygen), and air [18]. Helium is a suitable gas for this purpose because of its physical properties such as the small diameter of gas molecules and, thus, long mean free path compared to other gasses [19]. The most important advantages of plasma surface modifications are the ability to change the surface properties selectively, improving biocompatibility, and mimicking the local surface environment without altering the bulk attributes. It also enhances physicochemical properties and optimizes the bifunctionality [20].

The contact angle method was used to understand the changes in surface wettability. Meghdadi *et al.* (2019) reported an increase in the hydrophilicity of the cold atmospheric plasma-treated Poly (Ɛ-caprolactone) (PCL) surface using the water contact angle measurement [12].

In another study, Chiper *et al.* (2011) compared helium and argon plasmas on Polyethylene Terephthalate (PET) surfaces. They reported following He-DBD plasma treatment, only topographical modifications of polymer treated samples were observed, while polymer surfaces exposed to Argon-DBD plasma presented an increased roughness and, moreover a change in the water contact angle of PET films [21]. This fact also shows a considerably increased hydrophilicity induced by plasma. The results of the present study (Fig. 4) showed that there is a decrease in contact angle and increase the hydrophilicity for the cold atmospheric plasma-treated sample. This confirmed the availability of the glass surface for lysostaphin. This phenomenon was also confirmed with the results of the topographical investigations performed by the AFM technique (Fig. 3). In this regard, intensive surface modification without any structural bulk change was detected, which is in accordance with the results of Preedy *et al.* (2014). They have used He and He/O2 cold atmospheric plasma to surface modify ultra-high molecular weight polyethylene (UHMWPE). They showed that when UHMWPE was treated by cold atmospheric plasma, the surface becomes hydrophilic, and the adhesion of proteins and cells to the surface of the implants improved. This method stimulates the osseointegration process and facilitate the bone adhering to the implanted device and improves the function of the implant [22]. Moreover, to our knowledge, there is no data available on the immobilizing antimicrobial agent on surfaces using cold atmospheric plasma technique treatment. The interaction between lysostaphin and nano modifications of surfaces following treatment with cold atmospheric plasma is shown and confirmed by Fig. 3.

In conclusion, we used a helium atmospheric plasma jet on the glass surfaces that increased surface topography and hydrophilic surfaces, which could absorb lysostaphin and preserve its bioactivity.

Considering the magnitude of pathogen-related complications due to growth, persistence, and spread of pathogens on different surfaces, more attention should be paid to treat surfaces by coating the effective highly active, stable, and environment-friendly antimicrobial agents. This study has shown that the immobilized lysostaphin on the glass surface is biologically active and could eradicate pathogenic *S. aureus*, a significant cause of nosocomial infection.

[DOI: 10.29252/JoMMID.7.4.132]

ACKNOWLEDGMENT

We would like to thank Ms. Melika Amya for her assistance.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

REFERENCES

1. Miao J, Pangule RC, Paskaleva EE, Hwang EE, Kane RS, Linhardt RJ, et al. Lysostaphin-functionalized cellulose fibers with antistaphylococcal activity for wound healing applications. Biomaterials. 2011; 32 (36): 9557-67.

2. Pangule RC, Brooks SJ, Dinu CZ, Bale SS, Salmon SL, Zhu G, et al. Antistaphylococcal Nanocomposite Films Based on Enzyme− Nanotube Conjugates. ACS nano. 2010; 4 (7): 3993-4000.

3. Kusuma CM, Kokai-Kun JF. Comparison of four methods for determining lysostaphin susceptibility of various strains of *Staphylococcus aureus*. Antimicrob Agents Ch. 2005; 49 (8): 3256-63.

4. Shah A, Mond J, Walsh S. Lysostaphin-coated catheters eradicate *Staphylococccus aureus* challenge and block surface colonization. Antimicrob Agents Ch. 2004; 48 (7): 2704-7.

5. Sasai Y, Kuzuya M, Kondo S-i, Yamauchi Y. Cold plasma techniques for pharmaceutical and biomedical engineering: INTECH Open Access Publisher; 2011.

6. Dyer J, Grosvenor A. Protein Fibre surface modification. NATURAL DYES. 2011: 111.

7. Recek N, Jaganjac M, Kolar M, Milkovic L, Mozetič M, Stana-Kleinschek K, et al. Protein adsorption on various plasma-treated polyethylene terephthalate substrates. Molecules. 2013; 18 (10): 12441-63.

8. Jose B, Antoci Jr V, Zeiger AR, Wickstrom E, Hickok NJ. Vancomycin covalently bonded to titanium beads kills *Staphylococcus aureus*. Chem Biol. 2005; 12 (9): 1041-8.

9. Aumsuwan N, Heinhorst S, Urban MW. The effectiveness of antibiotic activity of penicillin attached to expanded poly (tetrafluoroethylene) (ePTFE) surfaces: a quantitative assessment. Biomacromolecules. 2007; 8 (11): 3525-30.

10. Farhangnia L, Ghaznavi-Rad E, Mollaee N, Abtahi H. Cloning, Expression, and Purification of Recombinant Lysostaphin From Staphylococcus simulans. Jundishapur J Microb. 2014; 7 (5).

11. Shapourzadeh A, Rahimi-Verki N, Atyabi S-M, Shams-Ghahfarokhi M, Jahanshiri Z, Irani S, et al. Inhibitory effects of cold atmospheric plasma on the growth, ergosterol biosynthesis, and keratinase activity in Trichophyton rubrum. Arch Biochemistry Biophys. 2016; 608: 27-33.

12. Meghdadi M, Atyabi S-M, Pezeshki-Modaress M, Irani S, Noormohammadi Z, Zandi M. Cold atmospheric plasma as a promising approach for gelatin immobilization on poly (ε-caprolactone) electrospun scaffolds. Progress in biomaterials. 2019: 1-11.

13. Szweda P, Schielmann M, Kotlowski R, Gorczyca G, Zalewska M, Milewski S. Peptidoglycan hydrolases-potential weapons against *Staphylococcus aureus*. App Microbiol Biot. 2012; 96 (5): 1157-74.

14. Szweda P, Kotłowski R, Kur J. New effective sources of the Staphylococcus simulans lysostaphin. J BIOTECHNOL. 2005; 117 (2): 203-13.

15. Gallo J, Holinka M, Moucha CS. Antibacterial surface treatment for orthopaedic implants. Int J Mol Sci. 2014; 15 (8): 13849-80.

16. Wu JA, Kusuma C, Mond JJ, Kokai-Kun JF. Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. Antimicrob Agents Ch. 2003; 47 (11): 3407-14.

17.Hoffmann C, Berganza C, Zhang J. Cold Atmospheric Plasma: methods of production and application in dentistry and oncology. Med Gas Res. 2013; 3 (1): 21.

18.Hoffmann C, Berganza C, Zhang J. Cold Atmospheric Plasma: methods of production and application in dentistry and oncology. Med Gas Res. 2013; 3: 21.

19. Bárdos L, Baránková H. Cold atmospheric plasma: Sources, processes, and applications. Thin Solid Films. 2010; 518 (23): 6705-13.

20. Atyabi SM, Sharifi F, Irani S, Zandi M, Mivehchi H, Nagheh Z. Cell Attachment and Viability Study of PCL Nano-fiber Modified by Cold Atmospheric Plasma. Cell Biochem Biophys. 1-10.

21. Chiper A, Rusu G, Vitelaru C, Mihaila I, Popa G. A comparative study of helium and argon DBD plasmas suitable for thermosensitive materials processing. Rom J Phys S. 2011; 56: 126-31.

22. Preedy EC, Brousseau E, Evans SL, Perni S, Prokopovich P. Adhesive forces and surface properties of cold gas plasma treated UHMWPE.Collioid Surface A. 2014; 460: 83-9.

Cite this article:

Ehsani G, Fahmide F, Norouzian D, Atyabi SM, Ehsani P. Bioactivity Determination of Recombinant lysostaphin Immobilized on Glass Surfaces Modified by Cold Atmospheric Plasma on *Staphylococcus aureus*. J Med Microbiol Infect Dis, 2019; 7 (4): 132-137. DOI: 10.29252/JoMMID.7.4.132

[DOI: 10.29252/JoMMID.7.4.132]