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A XANES study of the antibacterial activity of silver ions against *Acinetobacter baumannii*

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Abstract: One of the most challenging antimicrobial-resistant Gram-negative bacilli to manage and cure is pathogenic *Acinetobacter baumannii*. Numerous bacterial species, including the tough *A. baumannii*, are strongly inhibited by silver ions. However, there is currently a scarcity of information regarding the mechanism of silver ions' bactericidal effect. The objective of this research was to use X-ray near-edge structure (XANES) spectroscopy to investigate the antibacterial activity of silver ions against *A. baumannii*. The local environment around silver ions and their bonding to specific spots in the biomass can be studied using this non-destructive technique. The obtained results demonstrated that the biomass sample of *A. baumannii* treated with silver included formation of silver bonding to -SH, -NH, and -OH groups, with Ag-N and Ag-O being the most dominant binding types. The presence of uniformly distributed silver at the bacterial cells as revealed by scanning electron microscopy (SEM) suggests that the majority of the silver ions bond to the outer cell membrane of *A. baumannii*. Accordingly, the antibacterial mechanism most likely involves silver ions connecting to locations in *A. baumannii*'s outer cell membrane as well as to the amino acids.

Keywords: Antimicrobial-resistance, Bacteria, XANES, Multidrug-resistance, Silver

1. Introduction

Since ancient times, Ag ions have been understood to be a powerful antibacterial agent. It has been utilized to inhibit bacterial growth in a variety of applications, including medicinal, biological, and hygienic ones. Due to its high effectiveness and the fact that it doesn't alter the color, flavor, or odor of water, it is also utilized in water supply systems, air purification, cosmetics, clothes, and a variety of home products (Yaha et al., 1992). The mechanism of antimicrobial role of silver ions has mainly been ascribed to the affinity of Ag ions towards electron donor groups containing nitrogen, oxygen and sulphur atoms (Slawson et al., 1992). However, the exact mechanism of silver toxicity on bacteria has not been yet fully understood. Several hypotheses are explaining the antibacterial activity of silver nanoparticle including: (1) release of silver ions denaturize proteins by bonding with sulfhydryl groups (Dibrov et al., 2002); (2) attachment of Ag-containing particles on bacteria and subsequent damage to bacteria (Urnukhsaikhan, 2021), (3) generation of reactive oxygen species (Lemire et al., 2013). Later results in the deterioration of the plasma membrane and the inactivation of proteins and enzymes (Randall et al., 2015). Many mechanisms have been proposed as potential causes of the lack of microbial resistance to silver ions. (May et al, 2021).

In the last decade the Gram-negative bacterium *Acinetobacter baumannii* has been intensively studied due to its resistance to many classes of antibiotics. *A. baumannii* is listed by the World Health Organization (WHO) as priority

pathogen (Bassetti et al., 2011). The bacterium causes serious pneumonia and infection of urinary tract, blood stream and wounds. Patients in intensive care units are particularly susceptible to bacteraemia and pneumonias brought on by ventilators (Goic-Barisic et al., 2023). Its multidrug resistance comes from the presence of resistance genes that could be easily switched under the antibacterial influence (Cochis et al., 2016). As an alternative to antibiotics, herbal and silver compounds were shown as promising antibacterial materials against *A. baumannii* (Hrenovic et al., 2013; Milenkovic et al, 2014; Tiwari et al, 2015; Kaskatepe et al., 2016). We have recently found that Ag-containing zeolite exerts bactericidal effect towards *A. baumannii* (Hrenovic et al., 2013; Milenkovic et al, 2014) which has been explained by a release of movable silver ions present in the zeolitic lattice. This result can be the starting point for creating new types of desiccants, which are mainly intended to be utilized in air conditioner filters. *A. baumannii* colonizes an air conditioner, and the cells disperse by airflow throughout the hospital room, directly infecting immunocompromised patients and even hospital staff members (Pustijanac et al., 2023).

Considering the importance of this issue we investigated the interaction of silver ions with *A. baumannii* biomass in order to get a deeper insight into the bonding of silver ions and accordingly in its bactericidal activity. The bonding of silver ions with the target sites (i.e, S, N or/and O atoms) in the bacterial cells was studied by an X-ray absorption near-edge structure (XANES) spectroscopy which gave basic information about the bonds of Ag in the Ag-treated biomass.

2. Materials and Methods

2.1. Bacterial strain

For experiments the strain of *A. baumannii* RUH 134 was chosen. This strain is used as a reference strain for globally distributed *A. baumannii* belonging to international clone 2 (IC2). The strain is of clinical origin and multidrug-resistant to the streptomycin, spectinomycin, tetracycline, gentamicin, kanamycin, neomycin, piperacillin, co-trimoxazole and sulfamethoxazole, but sensitive to ceftazidime, ofloxacin, imipenem, amikacin, tobramycin, minocycline, nalidixic acid and ciprofloxacin (Nigro and Hall, 2012).

2.2. Preparation of biomass

The bacteria were pre-grown on a nutrient agar (Biolife, Italy) for 16 h at 37±0.1 °C to obtain the culture in the log phase of grow. Dense biomass from the nutrient agar was suspended into the 1 cm^3 of 0.5 g $AgNO_3$ dm^{-3}. The suspension was sealed and incubated in darkness during 2 h at room temperature of 22 °C with shaking at 190 rpm to assure a complete mixing. The concentration of AgNO3 was chosen according to the reported bactericidal concentration of 2 g dm-3 in 30 min of contact against Escherichia coli (Matsumura et al., 2003). In the preliminary experiment, the selected concentration of 0.5 g AgNO3 dm^{-3} was proven bactericidal after 2 h of contact against the investigated strain of A. baumannii. After incubation, the biomass was firstly centrifuged at 10000 rpm for 5 minutes and washed with distillate water to wash out the unbounded silver on the surface of the biomass. The washed biomass was left to dry in the dry sterilizer at 50 °C for 24 h. After 2 h of treatment with silver ions, the count of viable bacteria cells on nutrient agar was below the detection limit (< 10 colony forming units per cm^3).

2.3. Preparation of biomass

L-alanine, histidine, DL-aspartic acid, cysteine, imidazole, sodium citrate and AgNO3 used for the preparation of reference compounds were of analytical grade and supplied by the Sigma-Aldrich. Ag-alanine (Ag-Ala), Ag-histidine (Ag-Hist) and Ag-cysteine (Ag-Cys) were prepared according to literature procedure (Gmelin, 1975) using 0.1 M solution of AgNO3 and solutions of the amino acids in the molar ratio 1:1. Ag-citrate (Ag-Cit) was synthesized by the procedure given by Djokic (Djokic, 2008) and Ag-imidazole (Ag-Imid) by Nomiya et al. (Nomiya et al., 1997). Fourier transform infrared spectroscopy confirmed that the prepared compounds are the reported Ag-complexes. All complexes were left in darkness, protected from the light prior to their further use.

2.4. Methods

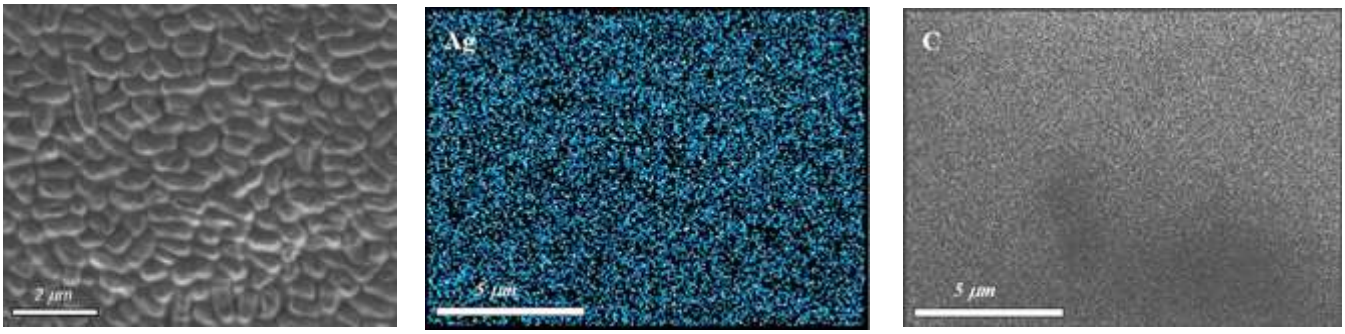
Elemental analysis of the biomass sample was performed by an energy dispersive X-ray analysis (EDAX) with an INCA Energy system attached on Zeiss Supra™ 3VP field-emission gun (FEG) microscope. Prior to the measurement, samples were sputtered with the 2 nm layer of carbon in order to avoid the excessive charging.

The Ag L_3-edge XANES (X-ray absorption near edge structure) spectra of the silver-treated *A. baumannii* and the reference compounds (standards) were recorded at the XAFS beamline of the ELETTRA synchrotron radiation facility in Trieste, Italy. A Si(111) double-crystal monochromator was used with about 0.4 eV resolution at the Ag L_3-edge. Higher-order harmonics were effectively eliminated by using a double-flat silica mirror placed at a grazing angle of 8 mrad. The intensity of the monochromatized beam in front and behind the sample was measured by 30 cm long ionization chamber detectors, filled with a gas mixtures: 30 mbar N_2, 1970 mbar He and 200 mbar N_2 and 1800 mbar He for the first and second ionization chamber, respectively. Biomass sample was put on a Kapton® adhesive tape to form a uniform layer. It was mounted on a sample holder in a chamber of the beamline that was filled with He during measurements to avoid absorption of the low energy X-rays used. The absorption spectrum was measured at room temperature in transmission detection mode. Spectra of the reference compounds: Ag-alanine (Ag-Ala), Ag-cysteine (Ag-Cyst), Ag-histidine (Ag-Hist), Ag-imidazole (Ag-Imid) and Ag-citrate (Ag-Cit) were measured under the same conditions. The exact energy calibration was established with an absorption measurement on argon gas as a secondary standard in transmission-detection mode. The Ar K-edge position was set to 3205.9 eV. The calibration spectra were taken before

and after the XAS measurements on the samples. Absolute energy reproducibility of the measured spectra was ±0.05 eV or better. The absorption spectra were measured in the interval from −300 eV to 180 eV relative to the Ag L_3-edge (3351 eV). In the XANES region, equidistant energy steps of 0.5 eV were used. The recorded spectra were analyzed with the IFEFFIT program package Athena (Ravel & Newville, 2005).

SEM analysis of the bacterial biomass showed densely deposited bacteria (Fig. 1a). The bacterial cells seem unaffected by the Ag-treatment, although were confirmed unviable by cultivation on nutrient agar. Silver and carbon are homogeneously distributed in the biomass sample (Fig. 1b). Bright spots (with diameter lower than 10 nm) seen in Fig. 1b (left) belong to Ag. The EDAX analysis showed that the concentration of Ag in the biomass is 1.3 wt. Ag %.

3. Results



(a) (b) (c)

Figure 1. Typical micrograph of the bacterial sample; b) EDAX elemental mapping of the sample showing Ag (left) and C distribution (right).

X-ray absorption spectroscopy is the method for studying local environment around the selected type of atoms. The local symmetry and the types of ligands around the studied atom both have an impact on how the absorption edge is shaped. It is feasible to determine the likely binding scenarios based on the "fingerprint analysis" by comparing the sample's XANES spectrum to the spectra of reference compounds with known oxidation states and ligand types (Bovenkamp et al., 2013; Vogel-Mikuš et al., 2012). The non-invasive nature of X-ray absorption spectroscopy makes it excellent method for examining the coordination geometry of various metal ions in biological systems as it causes minimal tissue disturbance (Lefevre et al., 2014).

The Ag L_3-edge XANES spectra of the Ag-treated biomass of *A. baumannii* and the reference compounds with known crystallographic structures are given in Fig. 2. Given that silver ions form complexes *via* sulfur, nitrogen, and/or oxygen atoms present in microbial cells as thiol, amine, hydroxyl, and/or phosphate groups, several Ag-complexes with amino acids have been chosen as the reference compounds. Silver, as a transition metal, exhibits a strong peak in its L_3 absorption edge, which is also known as the white line. In the case of Ag(I) compounds, the white line intensity can be enhanced through 5s – 4d hybridization and is related to the covalent bond between Ag and the ligand (Miyamoto et al., 2010). This makes it a distinguishing feature in determining the Ag coordination in a sample. Following observations by Bovenkamp et al. (2013), we also found that the white line of the Ag-treated biomass sample is more prominent and shifted approximately 1 eV towards higher energies compared to the white line in $AgNO_3$.

Further analysis of the local chemical environment of Ag ions in the Ag-treated biomass was done using a linear combination fitting (LCF) procedure. The LCF method gives

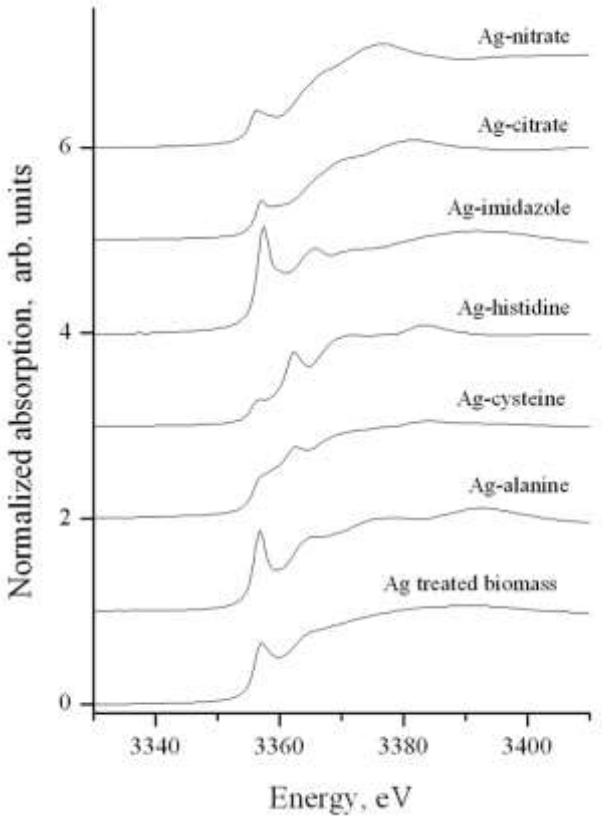


Figure 2. The Ag L_3-edge XANES spectrum of the Ag-treated A. baumannii biomass along with the spectra of $AgNO_3$ and the prepared Ag reference

compounds. The spectra are shifted vertically for clarity. The percentage contributions for the reference compounds in the linear combination fitting results.

It is worth noticing that spectra of several inorganic Ag compounds have also been recorded (Fig.3), however, their use in the LCF procedure did not give a satisfactory fit. This is in accord with the results of Bovenkamp et al. (2013) showing that the spectra of inorganic Ag compounds are not appropriate for using as references in fitting of XANES spectra of biomass samples.

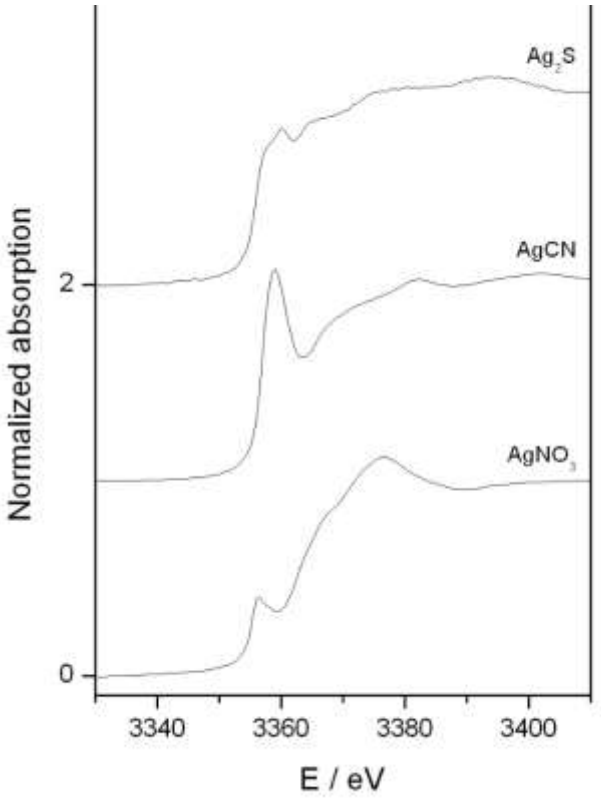


Figure 3. The Ag L_3-edge XANES spectrum of $AgNO_3$, AgCN, and Ag_2S. The spectra are shifted vertically for clarity.

The spectrum of the Ag-treated biomass of *A. baumannii* (Fig. 4) is best described as a linear combination of the Ag L_3-edge XANES spectra of Ag-Ala (21 %), Ag-Cyst (20 %), Ag-Imid (25 %) and Ag-Cit (34 %). The estimated error of the LCF is ±10 %, similar to that reported for results of the LCF analysis of XANES spectra of Ag-treated Gram-negative E. coli and Gram-positive S. aureus and L. monocytogenes (Bovenkamp et al., 2013).

The Ag-Ala was used as the reference compound for mixed bonding of Ag to both nitrogen and oxygen, Ag-Cyst was used for the Ag-S bonding, Ag-Imid and Ag-Hist for the Ag-N bonding, and Ag-Cit for Ag-O. Using the spectra of Ag-Hist and $AgNO_3$ in the LCF procedure did not yield acceptable fits. The LCF results also indicate that the entire amount of Ag is bound and no free silver ions are present in the biomass sample.

The results suggest that the Ag-S bonding participates to the lowest extent in the antibacterial mechanism (20%) (Fig. 3). Since Ag readily forms the Ag-S bond the interaction of silver ions with thiol (sulphydryl-SH) groups, which are associated with many important enzymes, it has usually been ascribed to the silver antimicrobial activity (Ito and Hirano, 1997). The results from this study indicate that the silver ions prefer O- and N-sites in A. baumannii biomass due to prevalence of Ag-O (34 %) and Ag-N bonding types (25%) as well as the mixed O,N-bonds (21%). However, it should be added that the sulphydryl groups are not present in significant amounts among amino acids. Only two (cysteine and methionine) from twenty amino acids have –SH groups. This may explain the fact that Ag-Cyst is not the dominant in the fit (Bovenkamp et al., 2013).

By comparing the obtained results to that reported for *S. aureus, L. monocytogenes* and *E. coli* (Bovenkamp et al., 2013), it can be concluded that the antibacterial activity of silver ions towards Gram-negative *A. baumannii* is similar to that found for Gram-negative *E. coli*. Mixed Ag-N and Ag-O binding was dominant in *E. coli*, which was attributed to the sites in nucleic acids in DNA. Accordingly, the antibacterial activity of Ag ions towards *E. coli* was explained by a disturbance of the DNA reproduction (Bovenkamp et al., 2013).

In this study, SEM analysis showed the presence of homogenously dispersed Ag (Fig. 1b) which could indicate that Ag ions mostly bind the sites in the outer membrane of *A. baumannii*. Like *E. coli*, *A. baumannii* forms a protective lipopolysaccharide layer with high amounts of –NH and –OH groups.

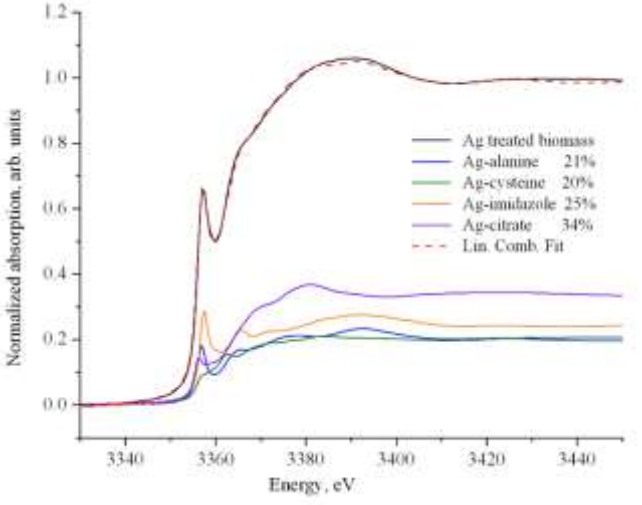


Figure 4. The Ag L_3-edge XANES spectrum of Ag-treated *A. baumannii* biomass (solid black line) and the best linear combination fit (dashed red line) obtained by the reference compounds (Ag-alanine, Ag-cysteine, Ag-imidazole, and Ag-citrate) plotted below. The relative amount of each component is given in parentheses.

4. Conclusion

The results of this study provide insight into the basic molecular mechanism of the antibacterial action of silver ions onto pathogenic multidrug resistant Gram-negative *A. baumannii*. According to the SEM and XANES studies silver ions are is uniformly distributed in the biomass, bound to –SH, -NH and –OH groups. *A. baumannii*'s outer cell membrane contains sites that include amine (-NH) and hydroxyl (-OH) groups that silver ions preferentially bind as well as the amino acids or DNA in the cell itself.

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Ispitivanje antibakterijskog dejstva jona srebra prema *Acinetobacter baumannii* XANES metodom

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Abstrakt: Jedan od najkompleksnijih gram-negativnih bacila otpornih na antimikrobne agense je *Acinetobacter baumannii*. Brojne vrste bakterija, uključujući rezistentan patogen *A. baumannii*, snažno su inhibirane jonima srebra. Međutim, trenutno postoji mali broj informacija o mehanizmu baktericidnog dejstva jona srebra. Cilj ovog istraživanja bio je korišćenje rendgenske spektroskopije u blizini elektronske granice (XANES) za ispitivanje antibakterijske aktivnosti jona srebra prema *A. baumannii*. Lokalno okruženje jona srebra kao i vezivanje za određene atome u biomasi mogu se proučavati korišćenjem ove nedestruktivne tehnike. Dobijeni rezultati su pokazali da uzorak biomase *A. baumannii* tretiran jonima srebra uključuje obrazovanje kovalentnih veza između srebra i -SH, -NH i -OH grupa, pri čemu su Ag-N i Ag-O najdominantnije veze. Prisustvo ravnomerno raspoređenog srebra u bakterijskim ćelijama utvrđeno je metodom skenirajuće elektronske mikroskopije (SEM) što upućuje na zaključak da se većina jona srebra vezuje za spoljašnju ćelijsku membranu *A. baumannii*. Shodno tome, antibakterijski mehanizam najverovatnije uključuje uspostavljanje veza između jona srebra sa grupama u spoljašnjoj ćelijskoj membrani *A. baumannii*, kao i sa aminokiselinama

Ključne reči: antimikrobna rezistencija; bakterije; XANES; rezistencija na antibiotike; SEM