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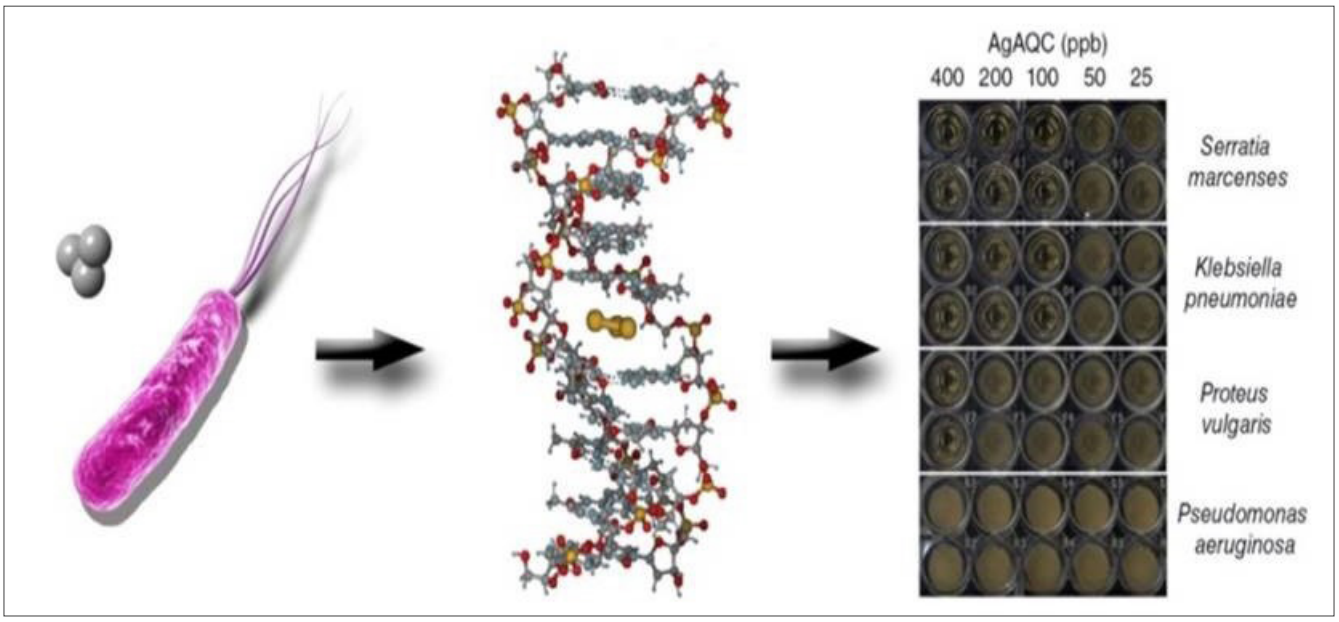
THE EFFECTS OF SILVER NANOCLUSTERS IN LIVING ORGANISMS*

Silver nanoclusters of 3 atoms were synthesized by a kinetically controlled electrochemical method and their interaction with DNA was studied by thermodynamic, kinetic and theoretical approaches. These silver nanoclusters intercalate into DNA inducing important structural changes in the DNA conformation. The changes in the DNA structure induce inhibition of DNA related enzymes such as topoisomerases, gyrases or restriction enzymes. Lastly, the bactericidal activity of these silver nanoclusters has been shown to open a new venue to be explored in the understanding of silver microbicidal properties.



The antibactericidal activity of silver has been known since ancient times. The existence of certain discrepancies about the reported activity of bulk silver, silver nanoparticles and Ag ion-based compounds have rendered their interpretation and application somewhat controversial [1]. Anyway, up to now it is quite clear the serious health problem that represents the widespread antibiotic resist-

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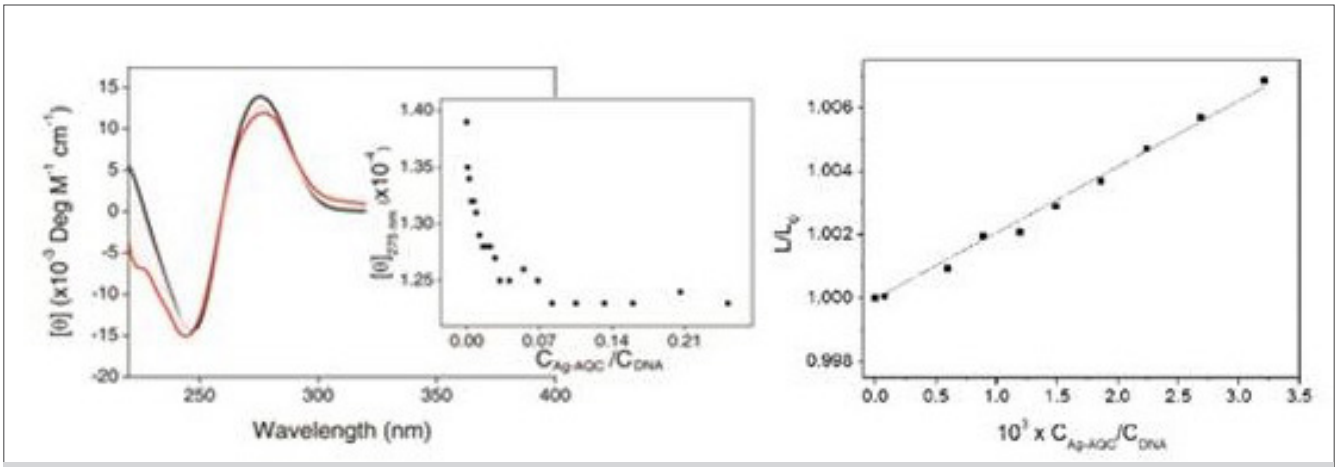

Fig. 1 - a) Circular dichroism spectra. C^0_{DNA}= 98 μM. Inset: Binding isotherm (molar ellipticity at λ = 275 nm); b) relative elongation ($L/L_0 = (\eta/\eta_0)^{1/3}$) of the Ag-AQC/DNA system *versus* $C_{Ag\text{-}AQC}/C_{DNA}$ ratio, C^0_{DNA} = 4x10^{-4} M. pH = 7.0, I = 0.1 M and T = 25 °C. Reproduced with the permission of [5]. Copyright 2016 Wiley

ance of human pathogens. Therefore, it is crucial to develop new substances able to replace the existing antibiotics. In this regard, silver nanoparticles have attracted much attention as bactericidal materials; their activity primarily depends on characteristics such as size, shape and surface or zeta potential [2].

The ability of some silver nanoparticles has been shown to depend also on both the capping agents and the release of silver ions [3]. Therefore, it would be really interesting to evaluate the bactericidal activity of this kind of subnanomaterials without the interference of coating agents. Hence, the first challenge to achieve consists of synthesizing such nanomaterials. The group by M.A. López-Quintela (University of Santiago de Compostela, Spain) has successfully accomplished this goal. The synthesis of atomic quantum clusters (Ag-AQCs) of small number of silver atoms (2 and 3) has been carried out by an electrochemical method [4], in which the kinetics of the process is very slow with low concentration of Ag ions in solution [5].

Ag-AQCs are uncharged species that do not migrate in electrophoresis gel experiments. These clusters

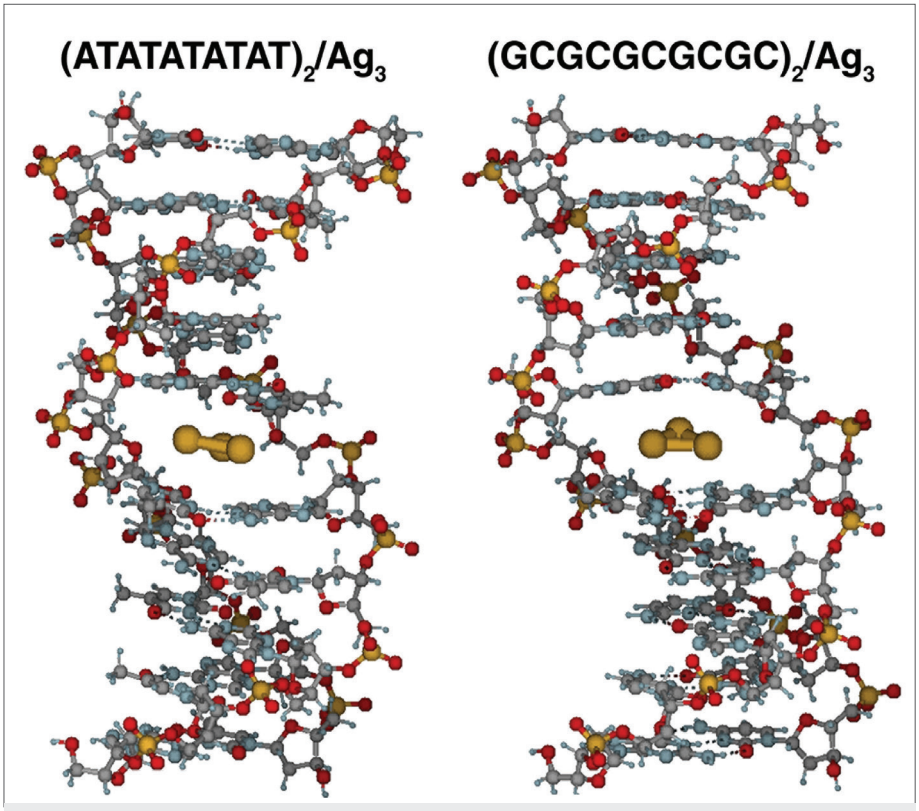

Fig. 2 - Minimum energy structures of the d(ATATATATAT)$_2$/AgAQC and d(GCGCGCGCGC)$_2$/Ag$_3$ complexes obtained by QM/MM calculation, showing the occurrence of intercalation of AgAQC between the 5th and 6th base-pairs. Reproduced with the permission of [5]. Copyright 2016 Wiley

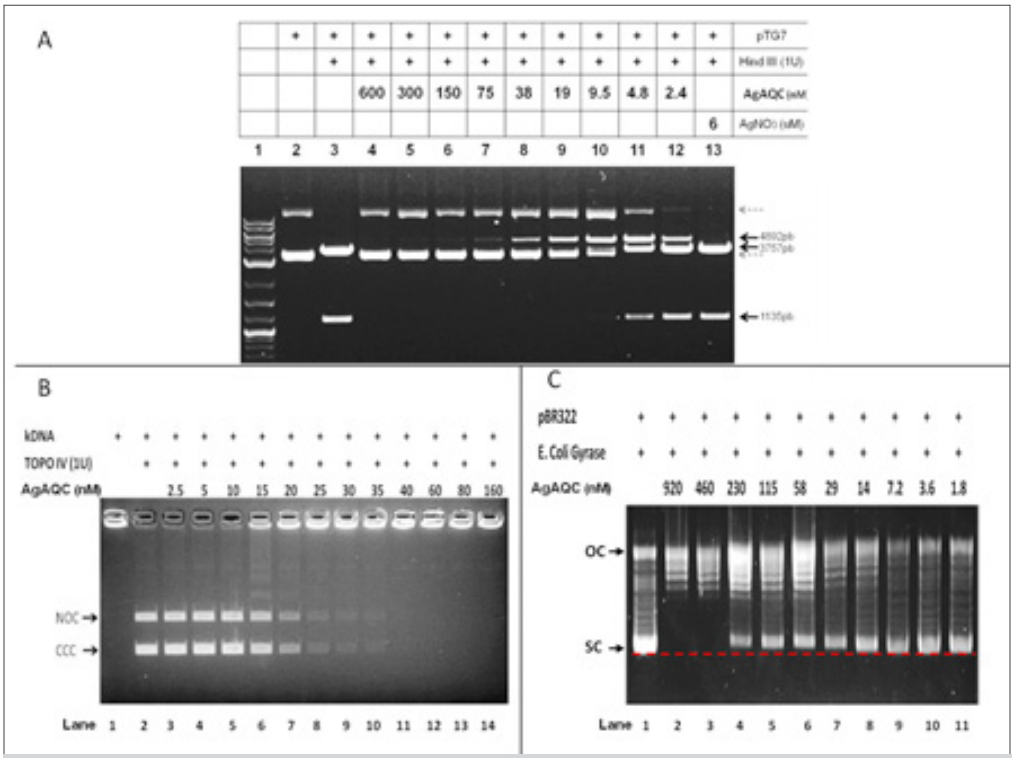

Fig. 3 - Agarose gel electrophoresis showing the inhibition by Ag-AQCs of a) restriction enzyme HindIII activity. The pTG7 plasmid (lane 2) was incubated for 30 min at 37 °C with one HindIII unit (lane 3) in the presence of nanomolar concentration of Ag-AQCs (lanes 4-12), or $AgNO_3$ (6 µM, lane 13), the reaction products were run on agarose gel. Continuous arrows indicate the theoretical products obtained after cut of the plasmid. Dotted arrows indicate the location of the plasmid with different supercoiling conformations. Lane 1, molecular weight markers; b) *E. coli* Topoisomerase IV decatenation activity. This gel contains ethidium bromide, which allows one to clearly resolve Topoisomerase IV generated nicked (NOC) and covalently closed circular (CCC) DNA. The kDNA networks are too large to enter the gel; c) *E. coli* DNA gyrase activity. The open-circular (OC) and supercoiled (SC) DNA are resolved and the relaxed DNA species are present as a Gaussian distribution of topoisomers, SC DNA has less mobility with increasing Ag-AQCs concentrations (dotted red line, lanes 4-9). The images are representative of at least three independent experiments. Taken from [13]

are very stable due to the large homo-lumo band-gap and, hence, they display very low tendency to undergo oxidation or reduction. The clusters size is (roughly) 300 pm height, as seen by AFM. Another important feature of these clusters is that they do not show any plasmon band, that is, there are no free electrons. This fact confers silver atomic quantum clusters certain properties very different from those of silver nanoparticles and also from bulk materials. In addition, the synthesized clusters are fluorescent in nature, with emission at 305 nm when excited at 230 nm. When the nanoparticles are formed, the fluorescent disappeared and the classic plasmon band emerged.

While the use of DNA as a scaffold in the synthesis of Ag nanoclusters is widely reported in the literature [6], little is known about the DNA binding of these nanomaterials, except some theoretical studies of the binding of silver clusters to the DNA bases [7]. Therefore, we found it interesting to fully characterize the interaction mode of these clusters with natural DNA. Absorbance and fluorescence titrations have enabled us to calculate the apparent binding constant by means of Hildebrand and Benesi equation [8], obtaining the values (5.0±1.3) $x10^4$ M^{-1} and (4.1±0.7) $x10^4$ M^{-1}, respectively, indicating good agreement between these two estimates [5].

The interaction of Ag-AQCs was also monitored by circular dichroism (A). The red and blue shifts (3 and 4 nm) in ellipticity at 275 and 247 nm, respectively, and the isodichroic point at 290 nm reveal profound changes in the structural properties of the DNA double helix as a consequence of Ag-AQCs interaction. The molar ellipticity at 275 nm as a function of the Ag-AQC/DNA concentrations ratio (Fig. 1A, inset) reached a plateau for 0.08 ratio, that is, saturation of DNA occurs at very small concentrations. To shed some light into the Ag-AQC/DNA interaction, viscosity experiments were also performed. Intercalating agents are able to cause an increase in the DNA contour length due to the local unwinding of the double helix [9]. Relative viscosity is related to the contour length according to the equation: $L/L_0 = (\eta/\eta_0)^{1/3} = 1 + \beta \times (C_{Ag\text{-}AQC}/C_{DNA})$, where L_0 and η_0 are the contour length and the vis-

cosity of the DNA alone, and L and η are those of the Ag-AQC/DNA system and β is the slope of the L/L_0 *versus* $C_{Ag\text{-}AQC}/C_{DNA}$ plot. Interestingly, the slope of the straight line described in this plot (Fig. 1B) is equal to 2. These results confirm intercalation as the binding mode, even if the slope achieved is greater than the values reported in bibliography (0.7-1.0) for classical intercalators such as acridines **[10]**, doxorubicin [11] or ethidium bromide **[9]**. This outcome can be easily understood if one considers that the covalent radius of Ag atom is approximately twice that of C atom (153 and 77 pm, respectively).

Intercalation was also confirmed by two-layer quantum mechanics/molecular mechanics calculations. Fig. 2 shows minimized energy structures of the geometric optimization of AgAQCs intercalated between the 5th and the 6th base-pairs of two double-helical decanucleotide duplexes $d(ATATATATAT)_2$ and $d(GCGCGCGCGC)_2$.

The kinetics of the AgAQC binding to DNA (reaction 1) was studied by stopped-flow technique in excess of DNA. The kinetic curves recorded were single exponential curves. The analyses of the relaxation times as a function of DNA concentration have enabled us to determine the formation, k_f, ($k_f = (1.19 \pm 0.08) \times 10^4\ M^{-1}s^{-1}$) and dissociation, k_d ($k_d = 0.15 \pm 0.06\ s^{-1}$) constants. These values yield the kinetic equilibrium constant $K = k_f/k_d = (7.9 \pm 0.7) \times 10^4\ M^{-1}$, in reasonable good agreement with the values obtained by absorbance and fluorescence titrations (see above):

$$AgAQC + DNA \underset{k_d}{\overset{k_f}{\rightleftharpoons}} AgAQC/DN \qquad (1)$$

The binding constant values are of the same order of magnitude than those obtained for other DNA intercalators **[10b, 12]**. Nevertheless, it should be noticed here that the rate constants differ respective the other systems mentioned above, the formation constant being two orders of magnitude smaller for AgAQC/DNA system. This could be easily explained taken into account that the silver nanoclusters are neutral species, whereas the rest of the intercalators are cationic in nature.

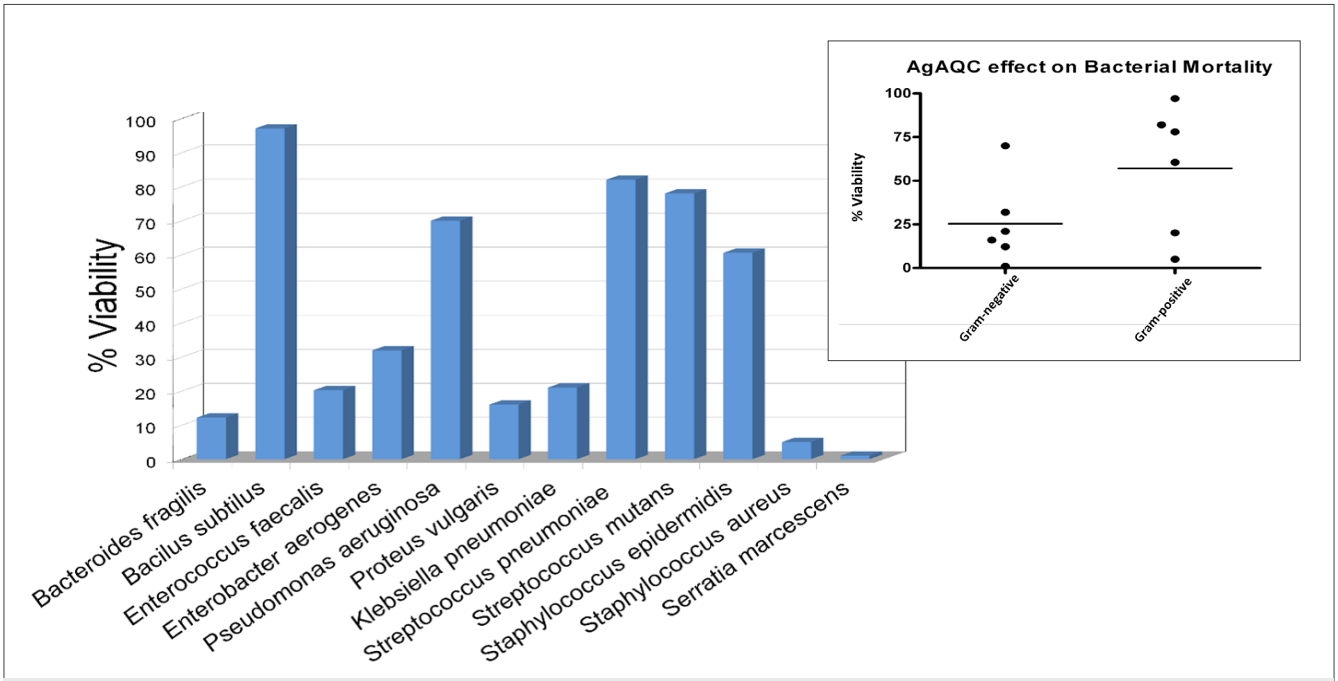

Fig. 4 - Bacteria isolated from clinical samples were treated for 1 h with Ag-AQC (83 ng/ml) and grown for 20 h. At the end of incubation, the absorbance at 600 nm was read, percentage viability is assessed as the percentage of absorbance of the Ag-AQCs treated bacteria versus control (vehicle). Inset: percentage viability of bacteria from different species grouped according to Gram-staining (horizontal lines, mean values of the group). Mean values of two independent experiments, taken from [13]

More interesting is the observation that the dissociation constant, k_d, of the AgAQC/DNA system is the lowest. This outcome is of great importance for therapeutic purposes, since slow dissociation rates mean long lifetimes in the intercalation site, which probably affects the DNA recognition, its replication and transcription.

With all this in mind, the inhibition of DNA related enzymes has been addressed, that is, DNA sequence dependent enzymes, such as the restriction enzyme Hind III, on one hand, and DNA topology dependent enzymes, such as Topoisomerase IV or *E. coli* gyrase, on the other hand. All of them were inhibited by silver nanoclusters at nanomolar concentration (Fig. 3). The effect of cationic silver at the same concentration range was negligible **[13]**.

Lastly, the bactericidal effect of silver nanoclusters was evaluated in several bacteria isolated from clinical samples (Fig. 4). It can be concluded that Gram positive bacteria are more resistant to silver nanoclusters than Gram negative (inset Fig. 4) **[13]**.

In summary, silver nanoclusters of 3 atoms were synthesized and their ability to intercalate into DNA has been studied in depth. The strong conformational changes induced in the DNA structure may be the responsible for the inhibition of DNA related enzymes. The bactericidal action of these silver nanoclusters has been demonstrated.

Taking into account that both, silver ions and silver nanoparticles, can lead to formation of silver nanoclusters in cells, the role played by silver nanoclusters in bacteria exposed to silver should not be discarded.

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Gli effetti di nanoclusters di argento negli organismi viventi

I nanocluster d'argento di tre atomi sono stati sintetizzati con un metodo elettrochimico cineticamente controllato e la loro interazione con il DNA è stato studiata mediante approcci termodinamici, cinetici e teorici. Questi nanocluster d'argento si intercalano nel DNA inducendo importanti cambiamenti strutturali nella conformazione del DNA stesso. I cambiamenti nella struttura del DNA provocano l'inibizione degli enzimi legati al DNA, come topoisomerasi, girasi o enzimi di restrizione. Infine, l'attività battericida di questi nanocluster d'argento ha dimostrato di aprire una nuova via da esplorare per comprendere le proprietà microbiocide dell'argento.