Monovalent Copper and Silver Ions Block DNA Polymerase Chain Reaction

Lea Moshkovich
Sami Shamoon College of Engineering

Oshra Saphier
Sami Shamoon College of Engineering

Stanislav Popov
Sami Shamoon College of Engineering

Yoram Shotland
Sami Shamoon College of Engineering

Eldad Silberstein
Soroka University Medical Center: Soroka Medical Center

Magal Saphier (✉ magal0564@gmail.com)
Sami Shamoon College of Engineering

Research Article

Keywords: Copper (I) ions, Antibacterial effect, DNA Polymerase, PCR, ATP, Enzyme inhibitor

Posted Date: June 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-543661/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Monovalent Copper and Silver Ions Block the DNA Polymerase Chain Reaction

Keywords
Copper (I) ions, Antibacterial effect, DNA Polymerase, PCR, ATP, Enzyme inhibitor

Abstract
In the present study we present the dramatic effect that monovalent copper ions (Cu(I)) have on the DNA polymerase chain reaction, and the moderate effect which monovalent silver ions (Ag(I)) have on it. Our research utilizes the commercial Polymerase Chain Reaction (PCR) system: in anaerobic conditions, in the presence of less than 0.1 μM of Cu(I) ions or in the presence of less than 10 μM of Ag(I) ions, the PCR system was entirely shut down.

Under the same conditions, 1 μM of divalent copper ions (Cu(II)) ions shows only a minor effect, while 10 μM of divalent Ni and Zn ions shows no effect at all.

This finding can give some explanation for the strong antimicrobial activity of monovalent copper ions (Cu(I)) as well as Ag(I). Although the mechanism of this effect is not yet fully understood, we recently published results showing that under the conditions of acidic pH, an unfavorable carbon source, low molecular oxygen concentration and elevated temperatures, the antibacterial action of Cu(I) ions is boosted, with a 10^6 bacterial population eliminated in less than 1 min by 0.4 mM of Cu(I). Microscopy checking of E.coli morphology and light scattering testes showed mortality of bacteria with almost no lysis. These results suggest that rapid and lethal metabolic damage is the main mechanism of Cu(I)’s antimicrobial effect.

Declarations
**Introduction**

Copper (Cu) is an essential trace element for all living organisms; in high concentrations, however, it can exert a biocidal effect. Recently [1], we suggested that monovalent copper ions (Cu(I)) are the active factor in copper's antimicrobial activity [1]. Although the mechanism of Cu(I)'s antimicrobial effect is not yet fully understood, we showed that in conditions of acidic pH, an unfavorable carbon source and elevated temperatures boost the antibacterial action of Cu(I) ions. In less than 1 min, 0.4mM of Cu(I) eliminated a10^6 bacterial population; microscope morphology of E.coli showed mortality of bacteria with almost no lysis [2].

Silver is also known as a biocidal agent: especially monovalent silver ions (Ag(I)) [3]. Copper and silver elements have a similarity as coins elements and their monovalent ions have a similar electronic configuration (d^10). It is reasonable to assume that both ions have a similar biocidal mechanism.
Copper ions (Cu(II) and Cu(I)) are known to form reactive oxygen species (ROS) [4] that can damage bio molecules, including DNA and chromatin. This has been well-demonstrated in vitro with isolated DNA or chromatin, or by exposure of cultured mammalian cells to copper complexes with various agents [5,6]. In vivo, however, according to the literature, copper ions do not catalyze the formation of oxidative DNA damage [7,8,9].

That said, in living cells there are mechanisms to control the intracellular concentrations of copper ions [9]. ATP7b and CopApump excess copper out of the cytosol and into the periplasm [10]. Once in the periplasm, copper is subject to two other systems, CueO and CusCFBA, that assist CopA in controlling intracellular copper levels. CueO is a multi-copper oxidase that converts Cu(I) to Cu(II), a less-toxic form [11]. It seems that copper ions, especially Cu(I), are a major threat to the cell.

In aqueous solutions, copper in the monovalent state (Cu+, cuprous) is unstable compared to the common oxidation state of copper ion, its divalent state (Cu2+, cupric). Cu+ (aq) will disproportionate to Cu2+ (aq) and Cu0 (s), it rapidly reacts with molecular oxygen, and as a consequence, Cu+ (aq) concentrations are usually very low.

To achieve significant concentrations of Cu+ ions, a ligand, such as Acetonitrile [12], benzoic acids [13] and ATP [14] has to be added to the solutions. These ligands shift the existing equilibrium between oxidation states to the formation of two Cu+ ions from one Cu2+ ion and metallic copper. Furthermore, the molecular oxygen concentration must be low.

The synthesis of DNA molecules from deoxyribose nucleotides is catalyzed by DNA polymerase [15]. DNA polymerase enzymes are essential for DNA replication, during
which the DNA polymerase copies the existing DNA strands to create two new strands that match the existing ones. These enzymes catalyze the:

The polymerase chain reaction (PCR) is a commercial method widely used nowadays in molecular and medical biology to make several (thousands to billions) copies of a specific DNA segment. The method uses a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the rmophilic bacteria.

The goal of this paper is to measure the influence of Cu(I) on polymerase by showing its effects on the PCR system.

Materials and methods

Anaerobic conditions (described in our previous studies [1,2]):

Production of Cu⁺ ions in the experimental solution. Prior to the PCR experiments, Cu⁺ was produced, starting with deaerated aqueous solutions containing a mixture of CuCl₂ as the source for Cu²⁺ ions, metallic copper and 100 mM acetonitrile as a stabilizing ligand, according to the reaction:

\[
Cu^{2+} + Cu^0 + nCH_3CN \xrightarrow{\text{H}_2\text{O}} 2Cu^+ (CH_3CN)_n
\]

\[
K_{1\%CH_3CN} = 1 \times 10^2
\]

Each Cu²⁺ results into two Cu⁺ ions in solution.
**Injection of copper ions solutions via syringes.** Solutions containing Cu\(^+\) or Cu\(^{2+}\) ions were gently injected through a three-way syringe valve into the PCR medium. The water used as a component of the PCR reaction was heated and cooled in the PCR device prior to insertion into the reaction tube, to remove oxygen and provide an anaerobic environment.

**PCR (Polymerase chain reaction).** The key ingredients of a PCR reaction are Taq polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube along with the cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow the DNA to be synthesized.

In this study all the PCR experiments went through the following stages: (1) 98 °C for 10 min; (2) 95°C for 20 s; (3) 60 °C for 20 s; (4) 72 °C for 50 min; (5) Repetition of stages 2 to 4 35 times; (6) 72 °C for 5 min; (7) Cooling to 16 °C until the samples were analyzed.

The PCR experiments used Normal Solution Composition (NSC), which contains:

- Taq polymerase, primers, PDNA, PCR water, 0.2M dNTP (part of the Ready mix solution). Most of the PCR experiments, in addition to the NSC solution, contained 0.3% CH\(_3\)CN metal ions (as chloride salts) at final concentrations of from 80 to 0.08 μM.

In the present study we used as a template a 369bp DNA from a EGFP genome, produced from a pEPI-EGFP vector.

**Gel electrophoresis** was used to separate the DNA fragments based on size and charge in order to quantify the PCR productivity under various experimental conditions.

**DNA durability to oxidation** was measured on DNA segments (from plasmids cut by restriction enzymes) that were incubated with 8μMCu(II), Ni(II), Zn(II) and Cu(I) in anaerobic atmosphere at the PCR conditions as described before. The segments tested for changes on Gel electrophoresis technique.
Results

Comparison of the effect of copper ions concentrations on PCR reaction under anaerobic conditions. Fig 1 presents the electrophoresis results of PCR experiments in anaerobic conditions. The expected DNA product appears at 369bp (row 2 and 4 of Fig 1, positive and acetonitril controls).

Cu(II) ions at concentrations of $1\times10^{-4}$M or higher block DNA amplification; where concentrations are lower than $1\times10^{-6}$M, Cu(II) does not interfere with PCR at all and enables 100% DNA amplification, comparable to the control. Cu(I) by contrast has a prominent effect: At concentrations of $1\times10^{-7}$M Cu(I) ions (the lower limit of our capability), DNA amplification is blocked (Fig 1, row 13).

The effect of molecular oxygen. Fig 2 presents electrophoresis results of PCR experiments in aerobic conditions, in a range of Cu(I) and Cu (II) concentrations.

Comparison Fig 2 to Fig 1 clearly shows that maintaining anaerobic conditions allows Cu(I) to inhibit the Taq polymerase. In all examined ranges of concentration, the DNA production was blocked (in 0.8µM some activity was observed but in lower concentrations; at 0.08µM no activity was detected). Allowing molecular oxygen to interfere decreases the effect of Cu(I) and at a saturation of lower than 8 µM copper ions, the DNA amplification appear.

It is reasonable to assume that most of the Cu(II) effect is a consequence of partial reduction to Cu(I). In the aerobic environment Cu(I) oxidized very quickly to Cu(II), reducing the Cu(I) concentration. Moreover, the molecular oxygen reacting with Cu(I) must produce reactive oxygen species (ROS): it seems that the influence of oxidation stress is negligible compared to the Cu(I) effect.
The effect of silver ion concentrations on the PCR reaction. Fig 3 presents electrophoresis results of the PCR experiments. The expected DNA product appears at 369bp (row 2 and 4 on Fig 4, positive and acetonitril controls).

Ag(I) ions at concentrations of $1 \times 10^{-5}$M, or higher block DNA amplification.

Comparison of the effect of Cu(I) and Ag(I) to Cu(II), Ni(II) and Zn(II). Due to their location in the periodic table, nickel, copper and zinc have bivalence ions with a similar ion radius. The ions are similar in their electrostatic attraction, so if the effect of Cu(II) is mainly due to electrostatic attraction there should be a similar effect with Ni(II) and Zn(II). Fig 4 presents the electrophoresis results of PCR experiments comparing Cu(II) to Ni(II) and Zn(II).

Table 1 summarizes relevant results of the PCR experiments using electrophoresis; all experiments were repeated at least twice.

**Table 1: relevant results of PCR experiments**

| Experiment                                           | Digital Normalized signal | Signal at 369bp | SD |
|------------------------------------------------------|---------------------------|----------------|----|
| Ladder                                               | ~10                       |                | 0  |
| NSC (Positive control)                              | 100                       |                | 0  |
| NSC without Taq polymerase (negative control)       | 0                         |                | 0  |
| NSC +0.3% CH$_3$CN (Acetonitrile 0.3% control)      | 97                        |                | 7  |
| NSC +0.3% CH$_3$CN + Cu$^{2+}$0.8µM                 | 26                        |                | 14 |
| NSC +0.3% CH$_3$CN + Cu$^{2+}$0.08µM                | 108                       |                | 25 |
| NSC +0.3% CH$_3$CN + Cu$^{2+}$0.8µM                 | 6                         |                | 13 |
| NSC +0.3% CH$_3$CN + Cu$^{2+}$0.08µM                | 0                         |                | 8  |
| NSC +1% CH$_2$CN + Ag$^+$0.8µM | 0 |   |
| NSC +1% CH$_2$CN + Ag$^+$0. 8µM | 68 |   |
| NSC +0.3% CH$_2$CN + Ni$^{2+}$0. 8µM | 83 | 9 |
| NSC +0.3% CH$_2$CN + Zn$^{2+}$0. 8µM | 100 | 1 |

*NSC - Normal solution composition: Ready mix, Taq polymerase, primers, PDNA, PCR, water

Fig 4 and Table 1 clearly indicate that Cu(I) and Ag(I) are different from Ni(II) and Zn(II): the latter have hardly any or no effect on Taq polymerase. The results show that Cu(I) blocks PCR in concentrations 100 times lower than Ag(I). Therefore, it is reasonable to assume that most of the Cu(II) effect is a consequence of a partial reduction to Cu(I).

**DNA durability to oxidation.** To rule out DNA oxidation as the mechanism, DNA segments (from plasmids cut by restriction enzymes) were incubated with 8µM Cu(II), Ni(II), Zn(II) and Cu(I) under aerobic conditions. Fig 6 presents the electrophoresis results.

The results presented in Fig 5 indicate that we can rule out DNA oxidation as the mechanism in the time and concentration scale of the PCR experiments.

**Discussion**

In this study we showed that very low concentrations of monovalent copper ions($1*10^{-7}$M Cu$^+$) and low concentrations of monovalent silver ions ($1*10^{-5}$MAg$^+$) cause a complete deactivation of DNA polymerase.
This provides some clues about the mechanism of the bactericidal effect of Cu\(^+\) and Ag\(^+\) previously described by our group (1). The mechanism is not one of oxidative stress, even though monovalent copper ions (much more than bivalent iron ions) react with molecular oxygen (do not need hydrogen peroxide as do bivalent copper and iron ions) to generate active oxygen species (ROS), so that a Fenton-like reaction can begin without hydrogen peroxide [4]. If the mechanism was one of oxidative stress, we would expect that an aerobic atmosphere would enhance the effect. But the results (Fig 1 compared to Fig 2) show the inverse: an anaerobic atmosphere increases the effect. Copper ions, both monovalent and bivalent, do indeed generate reactive oxygen species (ROS) but the impact of the latter on the system is negligible compared to the main effect. Monovalent silver ions do not generate reactive oxygen species (ROS) and yet Ag\(^+\) has a bactericidal effect, apparently via a mechanism similar to that of Cu\(^+\).

Research articles that have examined the role of oxidative stress generated by copper ions found no damage to DNA in vivo [7,8,9]. At non-cytotoxic concentrations, copper ions inhibit the repair of oxidative DNA damage induced by visible light [8], the inhibition probably resulting from damage to enzyme function caused by Cu(I) rather than from oxidative stress. Figure 6 shows that the DNA plasmids maintain their weight and are not harmed by monovalent copper ions and molecular oxygen.

In fact, Cu(I) can serve as an antioxidant agent. Recent results [16] show that a Cu(I) complex with ATP reacts very rapidly with a methyl radical (CH\(_3\)) to produce Cu(II) and methane, terminating the radical chain:

3.
Indeed, there is a report [8] that shows that copper ions reduce the oxidative DNA damage caused by Fe(II) and H₂O₂.

Copper(I) ions do not undergo the disproportion reaction in the PCR medium that is expected in aqueous solutions. In our research we added Acetonitrile to the medium as a stabilized ligand. Recent results [16] show that ATP and Adenosine form a strong complex with copper(I) ions, and shift the disproportion reaction to the left:

\[
2\text{Cu}^+ \text{ATP} \rightleftharpoons \text{Cu}^{+2} + \text{Cu}^0
\]

In vivo it is reasonable to assume that ATP and other nucleic acids serve as stabilized ligands for Cu(I) inside the cell. Calculations [16] suggest that in the complex Cu(I)-ATP, the Cu(I) interacts with the base (adenine) and the phosphate groups causes the ATP to distort and fold, probably disrupting its function as a co-factor in the enzymatic system.

Ions such Zn²⁺ and Ni²⁺ have a negligible effect on the system; Cu²⁺ have some effect (fig 5). It is known that the system needs Mg²⁺ ions for proper operation of the enzyme, but it does not seem that in the tested concentrations, the partial exchange of Mg²⁺ions by Zn²⁺ and Ni²⁺ or Cu²⁺ has a significant effect. It is possible that all of the effect of Cu²⁺ is due to a reduction to Cu⁺ in a stabilized Cu⁺ environment. It was
mentioned in the literature that Cu(II) is rapidly reduced to Cu(I) by sulphhydryl ligands in solution, including glutathione and cysteine (17).

Previous article [1] demonstrates that Cu\(^{+}\) is in two order of magnitude a more potent antimicrobial agent than Ag\(^{+}\). In this study we founds that Cu\(^{+}\) blocks DNA polymerase chain reaction with concentration low in two order of magnitude from Ag\(^{+}\) concentration (fig 4), it support the assumption that the Cu(I) and Ag(I) antibacterial mechanism functions via enzymatic inhibition.

The assumption that the Cu(I) anti-bacterial mechanism functions via enzymatic inhibition is also supported by recent results [2] showing that elevated temperatures boost the antibacterial action of Cu(I) ions. In less than 1 min at 40\(^{\circ}\)C, 0.4mM of Cu(I) totally eliminated a 10\(^{5}\) bacterial population; by comparison, an experiment at 20 \(^{\circ}\)C left 10\(^{2}\) bacteria surviving after 1 min. Heat stimulates the metabolism, and permanent enzymatic inhibition is amplified by enzymatic activity. Furthermore, the result demonstrates that the antibacterial effect of Cu\(^{+}\) is higher when the E.coli utilizes glycerol as the sole carbon source compared to lactose and glucose. Bacteria adapted to glycerol as the sole carbon source are more vulnerable to the impact of enzymatic inhibition than bacteria that grow with glucose. Apparently the effect is not only via damage to cell division: if that were the case, lengthening generation time would minimize the effect. The results show the opposite.

Some articles have demonstrated the anti-cancer effects of copper oxide. In the most recent article [18], the Fe-doped CuO NPs were tested in vivo, resulting in complete tumor remission in multiple syngeneic subcutaneous mouse models. In cancerous cells the metabolic rate is much larger than in normal cells, so it's likely to be much more susceptible to the inhibitory effect of copper ions or more precisely to Cu(I)
ions. It seems that the Cu(II) from the CuO NPs, reduced by glutathione or other reducing agents to Cu(I) in the cancerous cells, are probably inhibiting the DNA polymerase.

We suggest that Cu$^+$ and Ag$^+$ acts by interfering with enzymatic metabolisms, such as DNA polymerase, via uncompetitive inhibition of the enzymes.

More relevant than ever this finding may suggest a possible role of Cu(I) producing system as an antiviral agent as well. However that concept is yet to be proven.

Conclusions

Our result show the dramatic influence of Cu(I) and Ag(I) on the replicative ability of DNA. Zn(II), Ni(II) and Cu(II) have minor effects compared to Cu(I) and Ag(I).

References

1. Magal Saphier, Eldad Silberstein, Yoram Shotland, Stanislav Popov and Oshra Saphier, "Prevalence of Monovalent Copper Over Divalent in Killing Escherichia coli and Staphylococcus aureus"; Current Microbiology, https://doi.org/10.1007/s00284-017-1398-4, 2017

2. Stanislav Popov, Oshra Saphier, Mary Popov, Marina Shenker, Semion Entus, Yoram Shotland and Magal Saphier; "Factors Enhancing the Antibacterial Efect of Monovalent Copper Ions", Current Microbiology. December 2019, p 1-8, https://link.springer.com/article/10.1007/s00284-019-01794-6 2019.

3. Jean-Yves Maillard & Philippe Hartemann, "Silver as an antimicrobial: facts and gaps in knowledge", Critical Reviews in Microbiology Vol 39 - Issue 4, 2013.
4. Mohamed Masarwa, Haim Cohen, Dan Meyerstein, David L. Hickman, Andreja Bakac and James H. Espenson; "Reactions of low-valent transition-metal complexes with hydrogen peroxide". *J. Am. Chem. Soc.*, 110, 13, 4293-4297, 1988.

5. Martha Patricia, Cervantes-Cervantes, J. Víctor Calderón-Salinas, Arnulfo Albores and José Luís Muñoz-Sánchez; "Copper increases the damage to DNA and proteins caused by reactive oxygenspecies". *Biological Trace Element Research* Volume 103, pages 229–248(2005).

6. Zara Molphy, Creina Slator, Chryssostomos Chatgilialoglu and Andrew Kellett; "DNA oxidation profiles of copper phenanthrene chemical nucleases"; *Front. Chem.*, 21 April 2015 | https://doi.org/10.3389/fchem.2015.00028

7. Maria C. Linder; "The relationship of copper to DNA damage and damage prevention in humans". *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, Volume 733, Issues 1–2, Pages 83-91, 2012.

8. Tanja Schwerdtle, Ingrit Hamann, Gunnar Jahnke, Ingo Walter, Constanze Richter, Jason L. Parsons, Grigory L. Dianov and Andrea Hartwig; "Impact of copper on the induction and repair of oxidative DNA damage". *Molecular Nutrition and Food Research*, Vol 51, Issue 2, p-201-10, 2007.

9. Lee Macomber, Christopher Rensing, James A. Imlay; "Intracellular Copper Does Not Catalyze the Formation of Oxidative DNA Damage in *Escherichia coli*". *American Society for Microbiology Journals*, https://doi.org/10.1128/JB.01357-06, 2007.

10. Christopher Rensing, Bin Fan, Rakesh Sharma, Bharati Mitra, and Barry P. Rosen; "CopA: an *Escherichia coli Cu(I)-translocating P-type ATPase". Proc. Natl. Acad. Sci. USA97:652-656. 2000.

11. Gregor Grass, Christopher Rensing: *CueO* is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* Volume 286, Issue 5, 2001.

12. Parker AJ, Macleod ID, Singh P "Electrochemistry of copper in aqueous acetonitrile". *J SolutChem* 10 (11): 757-774 doi.org/10.1007/BF00649487, 1981.

13. Magal Saphier, Ariel Burg, Shlomit Sheps, Haim Cohen and Dan Meyerstein; "Complexes of copper (I) with aromatic compounds in aqueous solutions"; *J. Chem. Soc., Dalton Trans*, 1845-1849, 1999.
14. Domeńech A et al. "Electrochemistry of copper complexes with polyaza[n] paracyclophanes. Influence of ATP as an exogen ligand on the relative stability of the Cu(II) and Cu(I) oxidation states"; Inorganica Chimica Acta 299: 238–246. doi.org/10.1016/S0020-1693(99)00506-X. 2000.

15. Chien A, Edgar D.B, Trela J.M. "Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus"; Journal of Bacteriology. 127 (3): 1550–7, 1976, doi:10.1128/jb.127.3.1550-1557.1976. PMC 232952. PMID 8432.

16. Ana Mesica, Israel Zilbermann, Magal Saphier, Guy Yardeni, Eric Maimon and Dan Meyerstein; "The Redox Aqueous Chemistry of CuII/I ATP", The 83rd Annual Meeting of the Israel Chemical Society, 2016, https://events.eventact.com/ProgramView2/Agenda/Lecture?id=169886&code=2451124

17. Gorren, A. C. F., A. Schrammel, K. Schmidt, and B. Mayr; "Decomposition of S-nitrosoglutathione in the presence of copper ions and glutathione", Arch. Biochim. Biophys. 330:219-228. 1996.

18. Hendrik Naatz, Bella B. Manshian, Carla Rios Luci, Vasiliki Tsikourkitoudi, Yiannis Deligiannakis, Johannes Birkenstock, Suman Pokhrel, Lutz Mädler,* and Stefaan J. Soenen "Model-Based Nano engineered Pharmacokinetics of Iron-Doped Copper Oxide for Nano medical Applications", Angew.Chem.Int.Ed.2020,59,1828–1836, 2020.
Figures

Figure 1

Example of gel-electrophoresis results of PCR experiments in anaerobic conditions. Normal Solution Composition (NSC) was used, with additions of Cu(II) and Cu(I) ions at final concentrations of between 80 and 0.08 µM.

Figure 2
Example of gel-Electrophoresis results of PCR experiments in aerobic conditions. Normal Solution Composition (NSC) was used, with additions of Cu(II) and Cu(I) ions at final concentrations of between 80 and 0.08 µM.

Figure 3

Gel-electrophoresis results of PCR experiments. Ag(I) ions added at final concentrations from 80 to 0.08 µM.

Figure 4
Gel-electrophoresis results comparison of the effect of 0.8µM Cu(II), Ni(II), Zn(II) on PCR reactions under anaerobic conditions.

Figure 5

Gel-electrophoresis results of DNA segments (from plasmids cut by restriction enzymes) that were incubated with 8µMCu(II), Ni(II), Zn(II) and Cu(I) under aerobic conditions.