GREEN SYNTHESIS OF COPPER-BASED NANOSTRUCTURES USING TANNIC ACID AND TESTING OF THEIR ANTIBACTERIAL PROPERTIES

Hana KŘÍŽOVÁ, Jana ROTKOVÁ

Technical University of Liberec, Faculty of Mechatronics, Informatics and Interdisciplinary Studies, Studentská 2, 461 17 Liberec
hana.krizova@tul.cz, jana.rotkova@tul.cz

Abstract:

In this study, copper-based nanostructures were prepared by the so-called "green synthesis" through a redox reaction between tannic acid and cupric ions of $CuSO_4 \cdot 5\ H_2O$ in water without a pH adjustment. The tannic acid is a hydrolyzed tannin present in the leaves and bark of many trees and plants; as an antioxidant it has a relatively strong reducing properties. The resulting colloidal solution had a large surface area with a meso- and nanoporous structure and its antibacterial properties have been researched with use of the Gram-negative bacteria *Escherichia coli*.

Keywords:

Copper, nanoporous structure, tannic acid, antibacterial properties.

1. INTRODUCTION

Copper, copper oxide and copper-based nanostructures offer promising applications in various fields such as biosensors, additives to improve thermal and electrical conductivity, photocatalysts for decomposition of dyes, antimicrobial surfaces, etc. These materials can be prepared by physical (microwave radiation or thermal decomposition), chemical (sodium borohydride, sodium sulfite) or biogen reduction of copper salts (using number of plant antioxidants). The size, composition and stability of resulting nanoparticles can be influenced by altering reaction conditions (e.g. temperature, pH, type and polarity of the solvent, concentration and strength of the reducing agent, concentration of cupric ions or presence of other substances). The preparation of copper-based nanostructures in water solution using reducing properties of tannins is one of the many options of their „green synthesis". These preparation methods are particularly environmentally friendly and cost-effective (much less expensive compare to working with silver nanoparticles, for example).

1.1 Antibacterial properties of copper

An intensive research of antibacterial properties of metal surfaces and metal nanoparticles is related to an increased resistance of pathogenic bacteria to antibiotics. The antibacterial properties of copper have been known since ancient times, when people noticed that the water in copper vessels spoils much more slowly. Copper salts are successfully used for spraying plants to protect them from mildew, metal surfaces containing copper act as bactericidal materials. The literature reports on various concentrations of copper nanoparticles with bactericidal capabilities (0.025 [1] – 10 mg [2] of copper nanoparticles in 1 ml), that are associated with a strain of bacteria, size of nanoparticles, concentration of bacteria in suspension, time of interaction and other additives or method used.

The Gram-negative bacteria are generally considered to be more dangerous pathogens due to the construction of their cell wall. Compared to the Gram-positive bacteria, their plasma membrane and thin layer of peptidoglycan are further covered by an additional lipopolysaccharide and a protein membrane. Number of possible mechanisms for bactericidal effect of copper were reported; the most important is the catalytic generation of free oxygen radicals and hydrogen peroxide based on the Fenton reaction that causes the oxidative stress. Oxygen radicals (mainly hydroxyl and hydroperoxyl radicals) attack and destroy cell structures and enzymatic apparatus, disrupt integrity of phospholipid membranes and metabolism of bacteria.

1.2 Tannic acid

Tannic acid is hydrolyzed tannin contained in the leaves and bark of many trees and plants (e.g. horse chestnut wood, oak, acacia, sumac). Tannins have been always used in traditional natural healing as part of plant extracts for its astringent, antioxidant and many biogenic properties. Since the last century tannic acid became a part of the European Pharmacopoeias as a chemically pure substance. Tannic acid has relatively strong reducing properties and is able to reduce copper nanoparticles from copper salt solution in an alkaline environment, such as the Fehling's reagent. Complex compound of tannin and copper (tannates) forming nanoporous structures are created in an acidic environment. These structures have a good capacity to release copper and act as an antibacterial agent.

2. EXPERIMENTAL PART

2.1 Material

Copper (II) sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$, 249.69 g/mol (Lach-Ner)
Tannic acid, $C_{76}H_{52}O_{46}$, 1 701.20 g/mol (Sigma-Aldrich)
Culture of bacteria *Escherichia coli* K-12
Tryptone soya broth (Oxoid), Tryptone soya agar (Oxoid), sterile saline solution

2.2 Devices

UV/VIS spectrophotometer UV-1600 PC (Mapada)
Zetasizer Nano ZS (Malvern)
Scanning electron microskope UHR SEM Ultra Plus (Carl Zeiss)
Mini-rocker shaker MR-1 (BioSan), incubator (Memmert), densimeter, vortex mixer

2.3 Methods

2.3.1 Preparation of copper-based nanostructures

Solutions of cupric ions (0.07 M) and tannic acid (0.018 M) were prepared in sterile saline. These solutions were diluted 10 and 100 times; 4.5 ml of each of the concentrations were mixed in the ratio 1:1 (tannic acid:cupric ions). After two solutions were mixed, a redox reaction immediately created complexes forming precipitates and aggregates of copper base.

2.3.2 Size and stability of aggregates

Size of generated aggregates was measured by Zetasizer Nano using light scattering. The zeta potential is a parameter for determining the stability of nanoparticle colloidal system. The mutual repulsion of nanoparticles depends on having either a large negative or positive zeta potential. The minimum value of the zeta potential in the system stabilized only by electrostatic repulsion forces is ± 30 mV.

2.3.3 UV/VIS spectra

UV/VIS spectra of the tannic acid, cupric ions and their mixtures were measured in the quartz cuvette of UV/VIS spectrophotometer in the range of 200-800 nm to monitor spectral changes associated with the formation of copper-based aggregates.

2.3.4 Test of antibacterial properties

Antibacterial properties of copper/tannin colloidal solution were tested on the Gram-negative bacteria *E.coli* using the modified test method ASTM E2149-01 (Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions). A frozen culture of *E. coli* was revived in Tryptone soya broth in an incubator at 37 °C during 24 hours, then it was diluted to a concentration of 10^8 CFU/ml using a densimeter. Further dilution was made to the concentration of 10^5 CFU/ml. Addition of 1 ml of the bacterial suspension to a concentration of 10^5 CFU/ml to 9 ml of copper/tannin mixture formed final colloidal suspensions (each of volume 10 ml) containing 10^4 CFU of *E.coli* with 20, 200 or 2000 µg of copper in 1 ml. Blank samples were prepared by mixing of a bacterial suspension with saline or different concentrations of tannic acid. Sterile centrifuge tubes with these suspensions were shaken for 1 hour and 5 hours on a shaker. Subsequently, 1 ml of each suspension was applied to the surface of Tryptone soya agar in Petri dishes (universal broth). After 24 h of incubation at 37 °C the number of colonies was compared with a control sample without copper/tannin colloidal solution.

2.4 Results

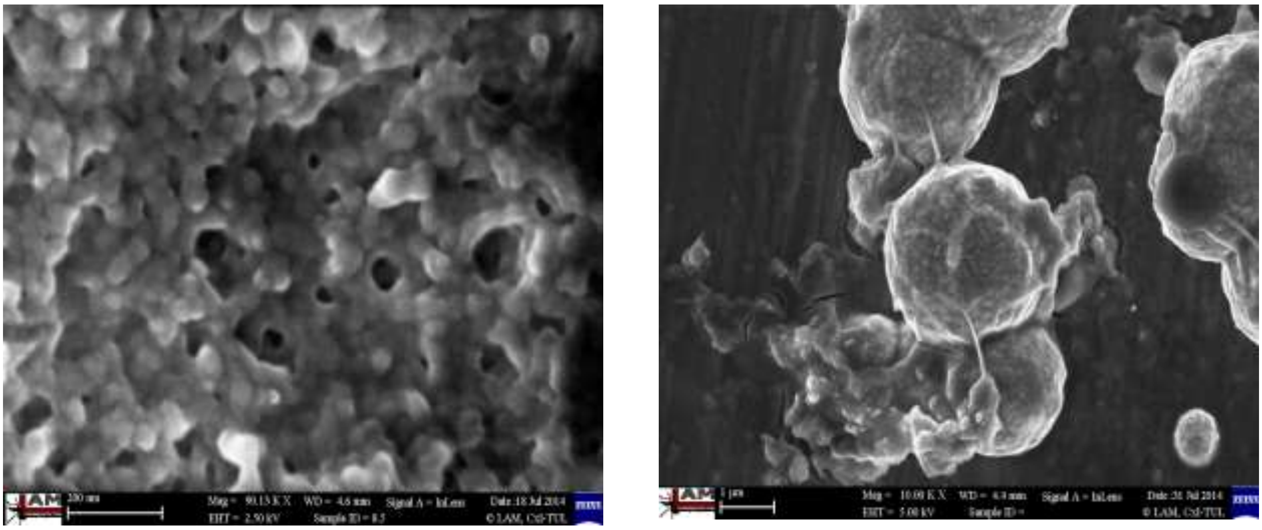

Fig. 1-2 Nanoporous structure of copper-based precipitates prepared via reduction by tannic acid

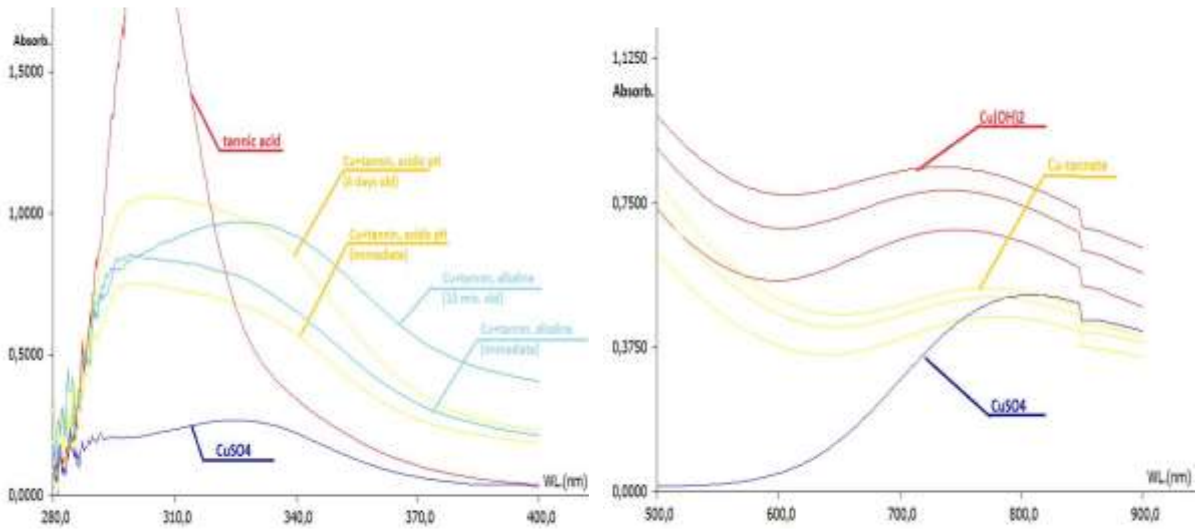

Fig. 3-4 UV/VIS spectra of tannic acid, cupric ions and resulting mixtures

Table 1: Size of copper/tannin aggregates (10 minutes old)

Concentration of tannic acid [mM/l]	Concentration of $CuSO_4 \cdot 5\ H_2O$ [mM/l]	Molar ratio (tannin : cupric ions)	Size [nm] (peak 1/ percentage)	Size [nm] (peak 2/ percentage
-	35.00	0:1	< 1/100%	-
8.8	-	1:0	1 – 1.9/100%	-
8.8	35.00	1:4	3 093/100%	-
0.88	3.50	1:4	370/90%	4 227/10%
0.088	0.35	1:4	74/80%	30/20%

Table 2: Size of copper/tannin aggregates (1 – 4 days old)

Concentration of tannic acid [mM/l]	Concentration of $CuSO_4 \cdot 5\ H_2O$ [mM/l]	Age of aggregates	Size (nm)	Zeta potential (mV)
0.3	1.2	4 days	2 800	- 8.7
0.3	1.2	3 days	1 735	- 9.3
0.3	1.2	2 days	980	- 9.6
0.3	1.2	1 hour	43	- 11.6

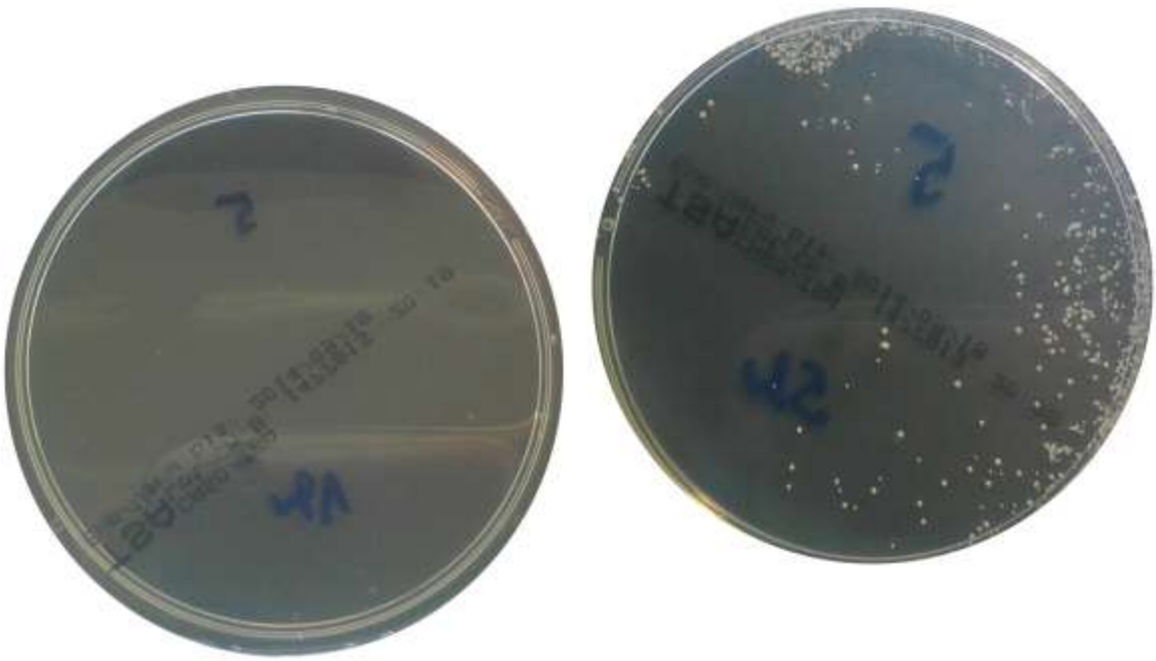

Fig. 5-6 Short term inhibitory effect of copper/tannin mixture of minimal copper content (20 μg/ml) on bacterial growth
Left: after 1 hour, right: after 5 hours of interaction with *E.coli*

Table 3: Number of CFU grown after 1 and 5 hours of interaction between 1 ml of bacteria *E.coli,* copper/tannin colloidal solution and tannic acid

Conc. of tannic acid in reaction mixture [mM/l]	Conc. of cupric ions in reaction mixture [mM/l]	Copper content in 1 ml of reaction mixture [μg]	CFU (1 hour)	CFU (5 hours)
-	-	-	3×10^4	3×10^4
8.0	-	-	0	0
8.0	32.0	2000	0	0
0.8	3.2	200	0	1
0.08	0.32	20	< 50	1.5×10^3
0.8	-	-	3×10^4	< 300
0.08	-	-	3×10^4	3×10^4
8.0	3.2	200	0	0
8.0	0.32	20	0	0

2.5 Discussion

The size of copper-based aggregates that were formed by a reaction of tannic acid and cupric ions increased with concentration of both components. As shown in Table 1, the size of these precipitates was increased with concentration of both starting solutions, even while maintaining their molar ratio 1:4 (tannic acid:cupric ions). It correlates with the finding that the size of nanoparticles increases with increasing tannin concentration in preparation of silver nanoparticles by reduction of tannins. [3]

As shown in the UV/VIS spectrum (Fig. 3-4), reaction of tannin with cupric ions creates tannin-copper complexes with maximum absorbance of about 790 nm and this absorbance value increases over time. The alkaline environment supports conversion of cupric salts to copper hydroxide with an maximum absorbance around 740 nm (maximum absorbance of copper sulfate itself is around 854 nm), and copper oxides are formed in the presence of a reducing agent. Fig. 3 shows the difference of waveform spectrum between copper hydroxide and tannin complexes with copper (spectrum difference is about 50 nm). The alkaline mixture shows changes in the spectrum after few minutes, while the waveform spectrum of copper-based complexes remains in the UV range after few days, the same as for a freshly produced. The differences in the spectrum of a mixture of copper/tannin with alkali and alkali-free and the pictures from scanning electron microscopy (Fig. 1-2) suggest that a slightly acidic environment of polyphenolic tannic acid supports more the formation of nanoporous aggregates of tannin with copper (tannates).

The low value of the zeta potential of copper-based aggregates (Table 2) indicates a low stability of resulting colloid, that corresponds to an increase in the particle size over time (from several tens of nanometers in size immediately after particle formation, up to a size of several micrometers during 4 days, while the colloid is "aging"; there is a further aggregation of the particles). Aggregates of resulting precipitates showed a meso- and nanoporous structure with a considerably dissected surface (Fig. 1).

The antibacterial effect of our copper/tannin mixture was tested on the Gram-negative bacteria *E. coli* using the method of immobilized antimicrobial agents under dynamic contact conditions. Table 3 shows that there was a complete inhibition of bacterial growth with concentrations of bacteria used (3×10^4 CFU in 1 ml) and with use of copper content of 200 μg in 1 ml or more. The copper content of 20 μg in 1 ml had only a short-term inhibitory effect, and after 5 hours of exposure there was an increase of bacteria growth, although 20 times less compared to the control sample (Fig. 5-6). Therefore, the copper concentration of 20 μg in 1 ml of suspension at contact conditions with the *E. coli* concentration of tens of thousands CFU in 1 ml can be considered the limit value.

Tannic acid alone showed an inhibitory effect on the bacterial growth while in prolonged contact with bacteria (after 5 hours), at the concentration of 0.8 mM/l (1.35 mg in 1 ml of suspension) or more. The concentration of 135 μg of tannin in 1 ml did not show any antibacterial effect, but a colloidal mixture with cupric ions at a concentration of 20 μg of copper in 1 ml showed a short-term inhibition of the bacterial growth.

3. CONCLUSION

This study involved experiments of copper-based nanostructures synthesized by green synthesis using tannic acid, their size and stability and their antibacterial effect on the Gram-negative bacteria *E.coli*. Resulting aggregates were sized from 43 nm (particles measured immediately) to 4227 nm (older aggregates), according to the age and concentration of tannic acid and cupric ions. The electron microscopy images shown greatly dissected surface with meso- and nanoporous structure of these aggregates. The copper/tannin suspension with bacteria *E.coli* in concentrations of 3×10^4 CFU in 1 ml showed not only a short-term inhibiting effect of copper on the growth of bacteria at the minimum copper content of 20 μg in 1 ml of the suspension, but also a longer-term inhibiting effect of only 0.8 mM tannin (after 5 hours of dynamic contact with bacteria in suspension). This proves that the *E.coli* bacteria is very sensitive to the copper/tannin mixture, that the natural tannic acid can act as an inhibitor of bacterial growth, as well as limit concentration values of both substances showed the synergetic effect of their antibacterial properties.

ACKNOWLEDGMENT

The paper has been elaborated with support of the grant project SGS 21066/115 provided by Faculty of Mechatronics of Technical University of Liberec and of the project LO1201 cofunded from the Ministry of Education, Youth and Sports as part of targeted support from the „Národní program udržitelnosti I“ programme.

LITERATURE

[1] SIVARAJ, R. et al.: Biogenic copper oxide nanoparticles synthesis using *Tabernaemontana divaricate* leaf extract and its antibacterial activity against urinary tract pathogen. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 133 (2014), p. 178 -181.

[2] NAIKA, H. R. et al.: Green synthesis of CuO nanoparticles using *Gloriosa superba* L. extract and thein antibacterial activity. *Journal of Taibah University for Science* (accepted 2014).

[3] KŘÍŽOVÁ, H., WIENER, J.*:* Synthesis of silver nanoparticles using condensed and hydrolysable tannins*. In conference proceeding: Nanocon 2013:* 16. - 18. 10. 2013. Brno, Hotel Voroněž, Czech republic, p. 600-605. ISBN 978–80–87294–47–5.