Red Microalgal Sulfated Polysaccharide–Cu$_2$O Complexes: Characterization and Bioactivity

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Cite This: https://dx.doi.org/10.1021/acsami.0c17919

ABSTRACT: The anion-exchange capacity of the cell-wall sulfated polysaccharide of the red microalga Porphyridium sp. can be exploited for the complexation of metal ions (e.g., Cu, Zn, Ag) to produce novel materials with new bioactivities. In this study, we investigated this algal polysaccharide as a platform for the incorporation of copper as Cu$_2$O. Chemical and rheological characterization of the Cu$_2$O–polysaccharide complex showed that the copper is covalently bound to the polysaccharide and that the complex exhibits higher viscosity and conductivity than the native polysaccharide. Examination of the complex's inhibitory activity against the bacteria Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and Bacillus subtilis and the fungus Candida albicans revealed a relatively high antimicrobial activity, especially against C. albicans (92% growth inhibition) as compared to the polysaccharide and to Cu$_2$O alone. The antibiofilm activity was also found against P. aeruginosa PA14 and C. albicans biofilms. An atomic force microscopy examination of the surface morphology of the complex revealed needle-like structures (spikes), approximately 10 nm thick, protruding from the complex surface to a maximum height of 1000 nm, at a density of about 5000/μm$^2$, which were not detected in the native polysaccharide. It seems that the spikes on the surface of the Cu$_2$O–polysaccharide complex are responsible for the antimicrobial activities of the complex, that is, for disruption of microbial membrane permeability, leading to cell death. The study thus indicates that the superior qualities of the novel material formed by complexion of Cu$_2$O to the polysaccharide should be studied further for various biotechnological applications.

KEYWORDS: Red microalga, Porphyridium sp., Sulfated polysaccharide, Cu$_2$O, Antibacterial activity, Antibiofilm activity

INTRODUCTION

The red microalgae, comprising a taxum of about eight genera, are morphologically the simplest of all the red algae. They are found in a range of different aquatic habitats, with some growing in fresh water and others growing in brackish or seawater. Reproduction is asexual, and in most species, the cells are brown as a result of their chlorophyll and phycocyanin contents. In studies of these microalgae, a great deal of work has been devoted to the genus Porphyridium, with both the ultrastructure and the conditions for growth being well documented. The cells of the red microalga Porphyridium sp. are encapsulated within a sulfated polysaccharide, whose external part dissolves in the medium, thereby increasing its viscosity. This sulfated polysaccharide is composed of 10 different sugars, of which the main ones are xylose, glucose, and galactose. The sulfated polysaccharide has a molecular mass of 5–7 × 10$^6$ Da and is negatively charged due to the presence of glucuronic acid and half-ester sulfate groups. The sulfate content in the polysaccharide varies between 6 and 7% w/v. Aqueous solutions of the polysaccharide are stable over a wide range of temperatures (30–160 °C), pH values (2–9), and salinities, and solution viscosity appears to be unaffected by changes in the environment. It has therefore been suggested that the sulfated cell-wall polysaccharide provides a buffer layer around the cells, protecting them not only against severe environmental conditions but also against bacteria, viruses, and fungi by virtue of its activity in maintaining cell humidity and in free radical scavenging under conditions of high light. The cell-wall polysaccharide may also contribute to cellular ionic regulation by selective cation binding. It is thus not surprising that the Porphyridium sp. cell-wall polysaccharide exhibits a variety of bioactivities, including antiviral, anti-inflammatory, antioxidant, and bio-lubricant activities. These unique qualities make it suitable for a variety of biotechnological applications.

Received: October 6, 2020
Accepted: January 25, 2021
variety of industrial applications, including those in the cosmetics, pharmaceutical, and food industries (as a gelling, thickening, and stabilizing agent).

By virtue of its negative charge and its anion-exchange capacity, it has been suggested that the polysaccharide can serve as a platform for metal incorporation as a means to produce new materials with synergistic metal–polysaccharide antimicrobial activities. For example, the complexation of the polysaccharide with zinc ions (as ZnCl₂) enhances its antibacterial activity compared with that of the native polysaccharide. Similarly, an association of copper ions (as CuCl₂) with the polysaccharide prevents biofilm formation by modulating bacterial adhesion. The applicative significance of the copper–polysaccharide interaction lies in the fact that microorganisms are extremely susceptible to copper, while copper is considered to be safe for external application on human skin, with a very low risk of adverse reactions. Indeed, numerous investigations have been conducted regarding the possible use of copper as an antimicrobial agent. These studies have shown that the bacterial toxicity of copper may be due to the displacement of essential metals from their native binding sites, interference with oxidative phosphorylation and the osmotic balance, and alterations of the conformational structure of nucleic acids, membranes, and proteins. In microorganisms, including microalgae, metals can cause the formation of reactive oxygen species that interact with lipids, proteins, and nucleic acids, resulting in their degradation. As a protective response, the synthesis of chelating agents, such as phytochelatin or exopolymers, is increased.

Studies of copper oxide—and other metals and metal oxides (silver, gold, palladium selenium, zinc oxide, and titanium dioxide)—in the form of nanoparticles have focused on their antifungal efficacy. In particular, the role played by the surface composition of the nanoparticles in their antimicrobial activity has been studied. For example, developed polyelectrolyte-coated titanias NPs with up to four layers of polyelectrolytes of alternating charge. A cationic outer layer (i.e., bare nanoparticles) was found to be a more effective antimicrobial than the anionic outer layer polyelectrolyte, probably by virtue of the increased adhesion of the cationic nanoparticles to the bacterial membrane. It has been shown that the antibacterial activity of Cu₃O₆-based nanoparticles derives from copper-induced plasmid DNA degradation in a dose-dependent manner in both Gram-positive and Gram-negative bacteria. Toxic effects of polymer-coated CuO nanoparticles have also been shown in the green microalga Chlamydomonas reinhardtii. In addition, it has been shown that Cu administered as a hydrid—Cu—chitosan complex was similarly highly effective against fungi and different bacterial strains even under anaerobic conditions (although Cu can be rapidly oxidized). Moreover, CuO has been widely used in hospitals as an antimicrobial agent, significantly reducing the occurrence of hospital-acquired infections and their costs. It is important to note here that—alongside its antimicrobial activity—copper is also an essential micronutrient for organisms. Physiologically, copper exists in the form of ions, and in fungi such as Candida albicans, it is a functional component of various enzymes and chaperones.

Recently, a different type of mechanism has been suggested for the antimicrobial activity of natural and bio-inspired materials that have a nano-spiked surface where the nanotopography spikes have been shown to physically rupture the microbial cell membrane of bacteria and fungi. The antimicrobial activity of such natural and synthetic spikes after adhesion to the microbial cell membrane has been attributed to a number of different factors, including the density and size of the spikes (ranging from 40 to 500 nm) and the rigidity of the microbial cell-wall (envelop architecture). However, the precise mechanism that leads to microbial cell death has not yet been elucidated conclusively.

The above studies provided the rationale for the current investigation in which we studied and characterized the bioactivity of a complex composed of the cell-wall sulfated polysaccharide of Porphyridium sp. and Cu₂O. It seems that the superior antibacterial and antifungal activities exhibited by the Cu₂O–polysaccharide complex were due to formation of spikes on the surface of the complex that led to mechanical damage to the microbial cell wall after adhesion of the complex to the microorganisms. It would thus appear that this complex has potential for development as a novel product that could be suitable for a variety of medical and cosmetic applications.

### EXPERIMENTAL SECTION

**Algal Growth and Polysaccharide Production.** Porphyridium sp. (UTEX 637) obtained from the culture collection of the University of Texas at Austin was grown in artificial seawater. Culture of the algal cells and isolation of the extracellular polysaccharide were performed as previously described. Briefly, the cells were grown in polyethylene sleeves in the appropriate medium. The cultures were illuminated continuously with fluorescent cool-white lights at an irradiance of 150 μE m⁻² s⁻¹ and aerated with sterile air containing 3% CO₂. Cells were harvested at the stationary phase of growth by centrifugation (CEPA, Carl Padberg Zentrifugenbau GmbH, Lahr, Germany). The supernatant containing the dissolved polysaccharide was collected and filtered using crossflow filtration to remove salts and other metabolites (MaxCell hollow fiber microfiltration cartridge, pore size 0.45 μm, membrane area 2.5 m²) and concentrated to 0.7% (w/v) polysaccharide. The resulting polysaccharide was sterilized in an autoclave and stored at 4 °C.

**Complex Preparation.** The Cu₂O–polysaccharide complex was prepared by directly adding Cu₂O (Fisher Scientific, Loughborough, UK) to 20 mL of a 0.7% (w/v) polysaccharide solution to give a final copper concentration of 500 ppm. The Cu₂O–polysaccharide complex was stirred gently with a magnetic stirrer for 24 h at room temperature and then sterilized by autoclaving.

**Copper Concentration.** The copper concentration in the Cu₂O–polysaccharide complex was evaluated by inductively coupled plasma optical emission spectrometry (SPECTRO ARCOS ICP-OES analyzer).

**Viscosity.** The viscosity of the polysaccharide solutions was determined with a Brookfield digital viscometer, 30 rpm at room temperature with a 31 cylindrical spindle (Brookfield AMETEK SC4-31).

**Conductivity.** The conductivity of the polysaccharide solutions was determined with a pH/mV/Cond./TDS/Temp. meter 86505 at room temperature.

**Cyclic Voltammetry.** A Metrohm 757 VA Computerize instrument was employed to obtain cyclic voltammograms of the Cu₂O–polysaccharide complex in an acetoniure solution at room temperature (25 °C) under a nitrogen atmosphere, with lithium perchlorate as the supporting electrolyte. A glass carbon working electrode, a platinum auxiliary electrode, and an Ag/AgCl reference electrode were also used.

**Rheology (G ', G ″).** Dynamic viscoelastic characterization of the Cu₂O–polysaccharide complex (0.7% w/v polysaccharide with 500 ppm Cu) was determined by the frequency dependence of the storage and loss moduli, G' and G ″. Measurements were carried out using a...
Complexes that the needle-like structures shown by the Cu₂O applied, the surface potential was recorded. It is important to note while in the second path, during which AC and DC voltages were oscillated mechanically and the surface topography was recorded, then with distilled water. Then, 10 spikes were counted by using Gwyddion and ImageJ software.

The Cu₂O—polysaccharide complex was prepared by directly adding Cu₂O to the polysaccharide and stirring the mixture gently with a magnetic stirrer for 24 h at room temperature. Values are means ± SD of three different experiments performed in triplicate. Polysaccharide concentration in all samples was 0.7% (w/v) and the pH was 4.5.

Table 1. Effect of the Addition of Copper on the Viscosity, Conductivity, and Zeta Potential of Cu₂O—Polysaccharide Complexes³⁻⁵

<table>
<thead>
<tr>
<th>sample</th>
<th>copper added (ppm)</th>
<th>viscosity (cP)⁶</th>
<th>conductivity (μS/cm)</th>
<th>zeta potential (mV)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharide</td>
<td>-</td>
<td>1830 ± 125</td>
<td>1.150 ± 20</td>
<td>−45 ± 3</td>
</tr>
<tr>
<td>Cu₂O—polysaccharide</td>
<td>250</td>
<td>1972 ± 220</td>
<td>2.532 ± 44</td>
<td>−47 ± 2</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2025 ± 110</td>
<td>2.857 ± 15</td>
<td>−48 ± 0</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>2328 ± 124</td>
<td>3.235 ± 20</td>
<td>−49 ± 3</td>
</tr>
</tbody>
</table>

³The Cu₂O—polysaccharide complex was prepared by directly adding Cu₂O to the polysaccharide and stirring the mixture gently with a magnetic stirrer for 24 h at room temperature. Values are means ± SD of three different experiments performed in triplicate. Polysaccharide concentration in all samples was 0.7% (w/v) and the pH was 4.5.⁴

Rheometer AR 2000 (TA Instruments) equipped with an extended temperature cell for temperature control and a stainless-steel parallel plate (d = 40 mm). The samples were held at room temperature for at least 20 min. After the temperature reached 25 °C, rheological tests were performed.

Fourier Transform Infrared Spectroscopy. Spectra were obtained on an IN10 FTIR ThermoFisher Microscope spectrometer equipped with a narrow-band liquid-nitrogen-cooled MCT (Mercury Cadmium Telluride) detector. Samples of the Cu₂O—polysaccharide complex and the polysaccharide were lyophilized at −55 °C for 24 h in 96-well plates. The spectra were recorded in three areas per sample in the range of 4000–650 cm⁻¹ at 2 cm⁻¹ resolution and 64 scans; the area of detection was 25 × 25 mm. The FTIR data were collected using OMNIC Picta software. Automatic baseline correction was used.

Scanning Electron Microscopy/Energy-Dispersive X-Ray Spectroscopy Analysis. The morphological properties of the Cu₂O—polysaccharide complex and the native polysaccharide were analyzed by SEM (FEI ESEM Quantia 200) at an accelerating voltage of 20 kV. In preparation for SEM scanning, 200 μL of samples was lyophilized at −55 °C for 24 h in 96-well plates. Then, the dried samples were attached to specimen holders with double-sided carbon tape and coated with a 20 nm layer of gold using the EMITECH K575x sputtering device (Emitech Ltd, UK).

Atomic Force Microscopy. Glass coverslips were immersed in 2.5 M HCl for 10 min and then rinsed first with ethanol (99.9%) and then with distilled water. Then, 10 μL of the Cu₂O—polysaccharide complex or the native polysaccharide was applied to the glass surface and dehydrated by autoclaving at 121 °C for 40 min. Topographical images of the pre-dried Cu₂O—polysaccharide complex and native polysaccharide were acquired on a Dimension-3100 microscope (Bruker). Samples were imaged at a scan rate of 0.5–1 Hz with a 512 × 512 pixel resolution in tapping mode. Several scans were carried out over a given surface area. Potential measurements via scanning Kelvin probe microscopy (SKPM) used an MFP-3D-Bio-inverted optical microscope system with an ARC2 controller (Asylum Research, Oxford Instruments). The images were recorded by SKPM in NAP mode with a two-pass method. On the first path, the cantilever was oscillated mechanically and the surface topography was recorded, while in the second path, during which AC and DC voltages were applied, the surface potential was recorded. It is important to note that the needle-like structures shown by the Cu₂O—polysaccharide complex in the AFM images were observed only under dry AFM conditions. AFM images were analyzed, and the needle-like structures (spikes) were counted by using Gwyddion and ImageJ software.¹⁻⁵

Microbial Cultures and Growth Conditions. The antimicrobial and antibiofilm activities of the Cu₂O—polysaccharide complex were tested on a variety of model microorganisms cultured as follows: Acinetobacter baumannii, Escherichia coli, and Pseudomona aeruginosa PA14 were cultivated for 24 h in Luria–Bertani (LB) Broth (Miller) (Sigma-Aldrich). Staphylococcus aureus was grown in DB Tryptic Soy Broth (TSB); soybean-casein digest medium. Bacillus subtilis was cultivated in LB medium (Difco Luria–Bertani medium, Lennox). C. albicans (ATCC 10231, supplied by the Clinical Microbiology Laboratory of Dr. Yossi Paitan, Meir Medical Center, Kfar Saba) was cultivated in Potato Dextrose Broth (PDB; HiMedia) for 24 h with shaking (120 rpm) at a constant temperature of 37 °C. For the antibiofilm study, P. aeruginosa PA14 was inoculated into AB trace Minimal Medium, supplemented with 30 mm glucose at a constant temperature of 37 °C.

Antimicrobial Activity. The antimicrobial activities of the Cu₂O—polysaccharide complex and of the native polysaccharide (in the form of a soft gel) against A. baumannii, P. aeruginosa PA14, E. coli, S. aureus, B. subtilis, and C. albicans were examined by determining the growth curves and viability of the microorganisms. Since Cu₂O is a photoactive material, the experiments were conducted in dark conditions. To determine the growth curves for the different species of bacteria and the fungus, 100 μL of polysaccharide (0.7% w/v), Cu₂O—polysaccharide complex (0.7% w/v), polysaccharide with 500 ppm of copper ions, or Cu₂O (500 ppm copper ions) solution was mixed with 900 μL of suitable medium and 10 μL of microbial culture at OD = 1. A sample of 200 μL of each combination was incubated with shaking in 96-well plates at 37 °C for 14 h for A. baumannii, P. aeruginosa PA14, E. coli, S. aureus, and B. subtilis or 48 h for C. albicans. The turbidity of the medium was measured hourly with a micro-plate reader (BioTek Instruments) at a wavelength of 600 nm. Growth inhibition was calculated from the following formula:

\[
\text{inhibitory index (％) = } \left(1 - \frac{\text{OD}_{\text{treatment}} - \text{OD}_{\text{control}}}{\text{OD}_{\text{controlblank}} - \text{OD}_{\text{controlblank}}} \right) \times 100
\]

where OD_{treatment} is the absorbance of the sample of the Cu₂O—polysaccharide complex or polysaccharide plus bacteria at t = 14 h (in the logarithmic phase of growth) or fungus at t = 48 h, OD_{controlblank} is the absorbance of the same sample without bacteria or fungus at the same time, OD_{control} is the absorbance of only LB or TSB (as mentioned above for each bacteria) and bacteria or PDB and fungus, and OD_{controlblank} is the absorbance of the same sample without bacteria or fungus at the same time. For cell viability determination, cells were incubated in test tubes containing 900 μL of LB, TSB, or PDB, as relevant, mixed with 100 μL of the Cu₂O—polysaccharide solution (0.7% w/v polysaccharide with 500 ppm copper ions) versus their respective controls (LB, TSB, or PDB alone or solutions of polysaccharide or copper ions). After 24 h, 100 μL of each sample was plated on an LB, TSB, or PDB agar plate after serial dilution and incubated overnight at 37 °C (under dark conditions). On the following morning, CFUs were counted.

Antibiofilm Activity. The morphological properties of the coatings and the biofilm structure were analyzed using SEM. Imaged biofilms of P. aeruginosa PA14 and C. albicans on Cu₂O—polysaccharide, polysaccharide, and Cu₂O surfaces were acquired using high-resolution (HR)-SEM. After 24 h of incubation, the samples were prepared for SEM studies as follows. After fixation in 2.5% buffered gluteraldehyde, the samples were subsequently dehydrated via an increasing serial ethanol gradient and immersed in a hexamethyldisilazane (HMDS)/ethanol gradient solution (25, 50, 75, 90, 95, and 100%). The treated specimens were air-dried for 4 h and in preparation for SEM scanning (JSM-7400F, JEOL), they were sputter-coated with a 20 nm layer of gold using an EMITECH K575x sputtering device (Emitech Ltd, UK).

https://dx.doi.org/10.1021/acsami.0c17919
ACS Appl. Mater. Interfaces XXXX, XXX, XXX–XXX
between the concentration of added Cu [from 0 ppm (native previously described by Guillaume Salek et al.57 In addition, been a reaction between the polysaccharide and Cu2O. To this spectra were used to determine whether there had indeed oc
copper does not change while in the complex, as was previously described by Guillaume Salek et al.57 In addi
to the other, to interact by the formation of H-bonds between the chains as was previously shown in polyelectrolyte solutions.55 The higher conductivities (up to 181% increase) of the Cu2O–polysaccharide complexes versus the native polysaccharide were probably due to the higher number of charged particles (Cu ions and polysaccharide). Our results showed that the native polysaccharide and the Cu2O– polysaccharide complexes exhibited similar values of the zeta potential (range of −45 to −49 mV), implying good stability.56 and probable complexation of Cu ions into the polysaccharide. The zeta potential of the Cu2O solutions (250/500/750 ppm Cu in buffer, pH 4.5) ranged from +20.5 to +22.9 as was previously described by Guillaume Salek et al.57 In addition, cyclic voltammetry analysis (Figure S1) of the Cu2O– polysaccharide complex showed that the oxidation state of copper does not change while in the complex, as was previously indicated.58 Since all three Cu2O–polysaccharide complexes exhibited higher viscosities and conductivities than the native polysaccharide and similar zeta potentials to that of the native polysaccharide, we decided to further develop and characterize the complex containing 500 ppm copper and 0.7% of the polysaccharide.

The dynamic viscoelastic properties of the polysaccharide and the Cu2O–polysaccharide complex were characterized in terms of the frequency dependence of the storage modulus G′(ω) and the loss modulus G″(ω) (Figure S2). The results obtained suggest weak gel behavior only for the polysaccharide solution (but not for solutions of the complex), in agreement with previous studies showing that a 1% (w/v) solution of polysaccharide behaves as a weak gel.18,59 The shift of the crossover position in the Cu2O–polysaccharide complex to lower than the measurable range (<0.1 rad/s) indicated a longer relaxation time of the chains and demonstrated that the solution behavior of the complex resembled that of a soft gel.

Spectrophotometric and Microscopic Analysis of the Cu2O–Polysaccharide Complex. Absorption coefficient spectra were used to determine whether there had indeed been a reaction between the polysaccharide and Cu2O. To this end, spectra of the absorption coefficients derived from the transmittance and reflectance spectra of the Cu2O–polysaccharide complex were compared to the spectra of the unmixed constituents, that is, polysaccharide and Cu2O (Figure S3). The difference between the measured and calculated curves for Cu2O suggests that some reaction did occur between the polysaccharide and Cu2O.

Similarly, the luminescence behaviors of solutions of the Cu2O–polysaccharide complex and the polysaccharide were significantly different from that of the Cu2O solution, with the latter solution exhibiting low luminescence intensity (Figure S4). The main difference between the spectra of the polysaccharide and the Cu2O–polysaccharide complex was observed in the infra-red range of the spectrum, manifested as a rise in the photoluminescence intensity of the complex at wavelengths longer than 800 nm. This rise may be attributed to the contribution of the added Cu2O since a similar rise was also observed in the photoluminescence spectrum of the Cu2O alone.

To elucidate how the copper binds to the polysaccharide, FT-IR spectroscopy was used (Figure 1). The transmission spectrum of the Cu2O–polysaccharide complex differed from that of the polysaccharide in that the former exhibited a new peak at 1180 cm−1, suggesting the formation of a new covalent bond, as has previously been shown.60,61 In both spectra (Cu2O–polysaccharide complex and polysaccharide), the broad band centered at 3260 cm−1 was assigned to O–H stretching vibrations and the weak signal at 2926 cm−1 to C–H stretching vibrations.62 Both the polysaccharide and the Cu2O–polysaccharide complex also exhibited a broad band at around 1220–1260 cm−1, which was assigned to sulfated ester groups (S=O)—also a characteristic component of the sulfated polysaccharides of other microalgae and indeed of other algae such as alginate in brown seaweeds.63,64 The surface morphologies of the polysaccharide and the Cu2O–polysaccharide complex were studied by SEM (Figure 2A,B). As can be seen from Figure 2, the structure of the polysaccharide is porous and fibrous. Differences can be seen in the quantity and in the size of the pores between the polysaccharide and the Cu2O–polysaccharide complex, with the complex having fewer entanglements and larger pores. In addition, in corroborration with the AFM results (see below), the Cu2O–polysaccharide complex showed a distinct morphology with bright, uniformly scattered needle-like structures (spikes). EDS—used to analyze the chemical compositions of the Cu2O–polysaccharide complex and the polysaccharide—showed five peaks corresponding to five major elements.
(Figure 2C,D), with the dominant gold peak being due to the gold coating. The main elements identified in the polysaccharide sample were carbon [61.87% (w/v)] and oxygen [30.87% (w/v)], as is to be expected for a sugar-containing polymer. In addition, sulfur [5.57% (w/v)] and small traces of copper [1.69% (w/v)], probably originating from the growth medium (which contains microelements including copper), were also evident. Figure 2D shows the elemental composition of the Cu$_2$O–polysaccharide complex detected by EDS (1—of the smooth area and 2—of the grainy area, i.e., the spikes, as seen in Figure 2B). Figure 2D-1 shows the elemental composition of the Cu$_2$O–polysaccharide complex detected by EDS. Again, the main elements identified in this sample were carbon [48.86% (w/v)] and oxygen [31.95% (w/v)] and a small amount of sulfur [8.06% (w/v)]. The main elements identified in the spikes (Figure 2D-2) were carbon [46.44% (w/v)] and oxygen [26.78% (w/v)] and a small amount of sulfur [12.53% (w/v)], indicating that the spikes were derived from the polysaccharide. As expected, the quantity of copper in the complex [11.13–14.24% (w/v)] was significantly higher than that in the polysaccharide. We assume here that the Cu is located on the surface of the complex.

To further study the surface topography and morphology of the Cu$_2$O–polysaccharide complex, AFM was used (Figure 3). The polysaccharide sample exhibited a generally smooth surface, but with some roughness throughout, that is, the raised structures were approximately 24.16–40.5 nm high and 1–10 nm wide. In contrast, the surface of the Cu$_2$O–polysaccharide complex was characterized by needle-like structures—spikes—that protruded up to 1000 nm above the surface and had a thickness of approximately 10–20 nm (Figure 3). The above morphologies were similar to those previously described for polysaccharide and Cu–polysaccharide complexes. Using both Gwyddion and ImageJ software,
the number of the spikes on the Cu$_2$O–polysaccharide complex surface was counted and found to be about 5000 ± 400 spikes/μm$^2$.

The above findings are in agreement with other studies showing that nano-topography spikes influence microbial viability after adhesion of a "nano-spiked" material to microbial cells as was previously shown for E. coli. It has also been shown that the rigidity of the microbial cells affects their susceptibility to mechanical rupture, for example, Gram-negative bacteria were more sensitive than Gram-positive bacteria, probably due to the cell wall architecture. Finally, it has been shown that the increased spike density and greater spike height promote the efficacy of the antimicrobial activity of the "nano-spiked" material. However, the precise mechanism leading to microbial cell death is not fully understood.

Antimicrobial Activity of the Cu$_2$O–Polysaccharide Complex. The antimicrobial activity of the Cu$_2$O–polysaccharide complex was evaluated (using turbidity measurements) in various model microbial pathogens, namely, the fungus C. albicans (generation time 82 min), the Gram-negative bacteria A. baumannii (generation time 48 min), P. aeruginosa PA14 (generation time 35 min), and E. coli (generation time 21 min), and the Gram-positive bacteria, S. aureus (generation time 30 min), and B. subtilis (generation time 28 min) (Table 2). Because of the dilution process of the assay (as described in the Experimental section), the final concentration of copper was 30 ppm, and polysaccharide concentration was 0.07% (w/v). The Cu$_2$O–polysaccharide complex almost completely inhibited the growth of C. albicans (91.7 ± 0.4% inhibition as compared with untreated cells), whereas Cu$_2$O alone exhibited low activity (about 15% inhibition), as previously reported for Cu$_2$O activity. The polysaccharide alone exhibited moderate activity in inhibiting C. albicans (35.8 ± 1.2% inhibition). For the various bacterial species, the Cu$_2$O–polysaccharide complex exhibited 72–78%

Table 2. Effect of the Cu$_2$O–Polysaccharide Complex on the Inhibition of Growth of C. albicans, A. baumannii, P. aeruginosa, E. coli, S. aureus, and B. subtilis

<table>
<thead>
<tr>
<th>time of exposure</th>
<th>microorganism</th>
<th>polysaccharide</th>
<th>Cu$_2$O</th>
<th>Cu$_2$O–polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h</td>
<td>C. albicans</td>
<td>35.8± 1.2</td>
<td>14.7± 1.9</td>
<td>91.7± 0.4</td>
</tr>
<tr>
<td>14 h</td>
<td>A. baumannii</td>
<td>40.1± 3.5</td>
<td>9.7± 2.9</td>
<td>78.5± 9.8</td>
</tr>
<tr>
<td>14 h</td>
<td>P. aeruginosa</td>
<td>45.9± 0.9</td>
<td>4.9± 6.5</td>
<td>78.1± 5.1</td>
</tr>
<tr>
<td>14 h</td>
<td>E. coli</td>
<td>49.2± 4.1</td>
<td>1.6± 1.8</td>
<td>75.0± 1.5</td>
</tr>
<tr>
<td>14 h</td>
<td>S. aureus</td>
<td>30.8± 5.6</td>
<td>20.9± 8.9</td>
<td>72.9± 7.1</td>
</tr>
<tr>
<td>14 h</td>
<td>B. subtilis</td>
<td>29.6± 2.9</td>
<td>15.6± 2.7</td>
<td>79.7± 2.5</td>
</tr>
</tbody>
</table>

The Cu$_2$O–polysaccharide complex contained 0.07% (w/v) polysaccharide and 30 ppm copper. Values in the table are means ± SD of three different experiments performed in triplicate.

Figure 3. AFM surface topography and 3D images of the polysaccharide and the Cu$_2$O–polysaccharide complex showing the smooth surface of the polysaccharide and the needle-like structures of the Cu$_2$O–polysaccharide complex. The Cu$_2$O–polysaccharide complex contained 0.7% (w/v) polysaccharide and 500 ppm copper.
inhibition. Cu2O alone did not inhibit bacterial growth, and the polysaccharide alone demonstrated only moderate inhibition (reduction of about 30−49% in growth for the bacteria, respectively, as compared with untreated cells). This difference in inhibitory activity between the Gram-negative and the Gram-positive bacteria may be due to their different cell wall compositions and structures.69

To investigate the bactericidal effect of the Cu2O−polysaccharide complex, the viability of the fungal and bacterial cells was determined in a CFU assay. Cells were inoculated on agar plates and allowed to grow at 37 °C. After an overnight incubation with the Cu2O−polysaccharide complex or its controls (growth medium alone or polysaccharide or Cu2O solutions alone), CFUs were counted and cell viability was determined in comparison with the control values (growth medium plus polysaccharide or Cu2O). The results show that the most effective treatment against C. albicans was that of the Cu2O−polysaccharide complex (Figure 4), with about 3% viable cells after the treatment as compared with ~45% viable cells with polysaccharide alone or the copper oxide alone. Similarly, low cell viabilities were previously reported for Cu2O.70,71 For all bacteria, no or minimal bacterial growth was detected. Thus, it seems that the Cu2O−polysaccharide complex not only inhibited fungal and bacterial growth but also caused cell death. A few studies have reported on the activity of Cu2O against bacteria and fungi. One of those studies showed relatively high antifungal activity of Cu2O−Cu nanoparticles/alginate (30 ppm Cu) against Neoscytalidium dimidiatum, but only after a long incubation period of 8 days.24 In a different study, an additive effect was found for chitosan-copper against C. albicans, Candida parapsilosis, E.coli, and P. aeruginosa when higher copper concentrations were used (>1000 ppm).72 Relatively high activity was also observed with PET/Cu2O@ZrP nanosheets having a high Cu content (186,200 ppm) against S. aureus, E. coli, and C. albicans.73

In summary, it is evident that the antifungal and antibacterial activities of the Cu2O−polysaccharide complex are more potent than those of the polysaccharide or Cu2O alone. The viability test also showed that the Cu2O−polysaccharide complex not only inhibited microbial growth but also reduced the number of viable cells. The low inhibitory effects of the polysaccharide alone against all the investigated bacterial species (A. baumannii, P. aeruginosa PA14, E. coli, S. aureus, and B. subtilis) might result from mass transport limitation of nutrients to the cells. The strongest effect was obtained for the Cu2O−polysaccharide complex, which caused 91% inhibition of C. albicans growth after 48 h and 97% cell death after 24 h. It seems that the antimicrobial potency of the Cu2O−polysaccharide complex versus the polysaccharide or Cu2O alone is probably due to the spikes on the surface of the Cu2O−polysaccharide complex.

Figure 4. Effect of the Cu2O−polysaccharide complex on the cell viability of C. albicans, A. baumannii, P. aeruginosa, E. coli, S. aureus, and B. subtilis cultures. The Cu2O−polysaccharide complex contained 0.07% (w/v) polysaccharide and 30 ppm copper. Values are the average of triplicate determinations. With the Anova test, all the results were significant compared to the control (p < 0.05). PDB, TSB, LB.

Figure 5. HR-SEM images of the Cu2O−polysaccharide complex, polysaccharide, and Cu2O surfaces in biofilm assays against C. albicans and Pseudomonas aeruginosa PA14. The microorganisms growing on clean glass surfaces served as the control. The Cu2O−polysaccharide complex contained 0.7% (w/v) polysaccharide and 500 ppm copper.
To investigate the antibiofilm activity of the Cu$_2$O–polysaccharide complex, C. albicans and P. aeruginosa PA14, used as model pathogens, were grown on clean glass surfaces in the presence and absence (as the control) of the Cu$_2$O–polysaccharide complex or the polysaccharide or Cu$_2$O alone. The Cu$_2$O–polysaccharide complex (dehydrated) on the glass surface demonstrated significantly improved microbial clearance as compared to a polysaccharide film and Cu$_2$O alone (Figure 5). In response to the Cu$_2$O–polysaccharide complex treatment, only a few individual cells were observed. Antibiofilm activity was less evident on the surface treated with the polysaccharide or Cu$_2$O alone (Figure 5).

**CONCLUSIONS**

In this study, we undertook a physicochemical and bioactivity characterization of a Cu$_2$O–polysaccharide complex. It was shown (by FT-IR) that in the Cu$_2$O–polysaccharide complex, the Cu ions were covalently bound to the polysaccharide. The complex exhibited higher viscosity and conductivity than the native polysaccharide, but zeta-potential and rheological properties were similar. Similarly, the complex displayed a porous fibrous structure and weak-gel-like behavior, similar to the native polysaccharide.

The Cu$_2$O–polysaccharide complex was effective against various microorganisms as compared to the polysaccharide or Cu alone. It almost completely inhibited C. albicans (91% inhibition) and presented significant inhibitory activity of biofilm formation against bacteria (P. aeruginosa) and fungi (C. albicans) as was seen in SEM images. The Cu$_2$O–polysaccharide complex was characterized by needle-like topographical protrusions (on AFM), which leads us to the conclusion that these structures are related to the complex’s antimicrobial and antibiofilm activities. Although the EDS–SEM studies indicated the existence of Cu on the surface of the complex, probably responsible for a cationic charge localized on the complex surface, the main antimicrobial effect may be attributed to the modified surface topography of the Cu$_2$O–polysaccharide complex, that is, to the spikes 1000 nm in height and 10–20 nm in width, at a density of about 5000 spikes/μm$^2$. It seems likely that the high density of the spikes coupled with their height can cause mechanical penetration of the microbial membrane, leading to disruption of membrane permeability and consequently to cell death, as has been shown previously.

The sulfated polysaccharide of Porphyridium sp. was used here as a platform for the incorporation of Cu$_2$O. The surface of the Cu$_2$O–polysaccharide complex was dramatically modified, yielding a new surface topography and hence new bioactivities. These results encourage further development of the unique Cu–polysaccharide complex as well as other metal–polysaccharide combinations toward the development of additional metal–polysaccharide complexes with potential antibacterial and fungicidal activities.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.0c17919.

Cyclic voltammogram of the Cu$_2$O–polysaccharide complex in acetonitrile; mechanical spectrum of the polysaccharide and the Cu$_2$O–polysaccharide complex; absorption coefficient spectra of polysaccharide, Cu$_2$O–polysaccharide complex, and Cu$_2$O alone versus wavelength; and photoluminescence spectra of the polysaccharide, Cu$_2$O–polysaccharide complex, and Cu$_2$O (PDF).

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**Notes**

The authors declare no competing financial interest.

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