Antibacterial and Antibiofilm Studies of Synthetic Copper(I)-containing Small Molecule Complexes Against *Streptococcus Mutans* in Vitro

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Abstract

Background

With the goal to develop high efficiency oral antimicrobial agents to prevent dental caries, a collection of copper(I)-containing small molecule complexes with different substitution were synthesized and characterized. In current research, we screened these complexes and explored their antimicrobial activities, especially against cariogenic bacteria *Streptococcus mutans*.

Methods

The characterization and minimum inhibitory concentrations of these complexes have been determined by NMR spectroscopy and standard broth microdilution, respectively. Biofilm formation and morphology were evaluated using crystal violet staining, MTT, confocal laser scanning microscope and scanning electron microscope. Finally, we tested the biocompatibility.

Results

Cu1 was screened and presented excellent performance, which suggested that the less lipophilic and less steric copper complexes would be effective toward the bacterial. **Cu1** also effectively inhibited the formation of *S.mutans* biofilm at its minimum inhibitory concentration.

Conclusions

This study demonstrates a high potential of copper(I)-containing small molecule complexes as broad-spectrum inhibitors to treat oral bacterial, especially for diminishing *S.mutans* biofilm formation.

1. Background

Dental caries, the most common oral diseases, is simultaneously one of the most common chronic illness [1, 2]. According to the statistical analysis of 328 diseases or injuries for 198 countries worldwide in 2016, the prevalence of dental caries was the highest[3]. In the process of caries formation, the colonization of pathogenic bacteria and the formation of biofilms are the main ecological factors[4]. *Streptococcus mutans* (*S.mutans*) acts as one of the major causative agents of dental caries. Its acid production, acid resistance, adhesion, ability to synthesize intracellular and extracellular polysaccharides(EPS), and biofilm formation are closely related to the occurrence and development of caries[5, 6]. In addition, certain pathogens can accelerate the disease progression[7].

Major biofilm management strategies are based on two approaches: physical removal and antimicrobial chemotherapy[8]. Chlorhexidine mouthwash is a conventional chemical agent for clinical plaque control, while long-term use can cause extrinsic tooth staining or reduction in human keratinocytes and fibroblasts[9, 10]. Furthermore, other oral care products such as triclosan or quaternary ammonium compounds are also at risk for bacterial resistance[10]. Therefore, it is extremely necessary to explore and develop additional effective drugs acting on several strains of oral pathogenic bacteria, especially exhibiting the inhibitory effect of *S. mutans* and its biofilm formation.

With metallopharmaceuticals playing a key role in therapeutic and diagnostic medicine, novel metal-complexes are ever-increasing in medical chemistry[11]. Until now, most efforts have been focused on the silver antibacterial agents [12–15], which exhibited significant antimicrobial properties at a relative high concentration of the silver ion. However, a large scale

use of silver complexes and nanoparticles triggers following bacterial silver resistance as well as argyria[16–18]. On the other hand, the copper-containing small molecule complexes was less explored[19, 20], even though the metal of copper has long been recognized as antimicrobial agent in drinking water treatment and transportation[21]. Very recently, Roland revealed that the small molecule copper-*N*-heterocyclic carbenes (NHCs) would be promising antibacterial agents, which displayed excellent performance for the inhibitory activity against *Listeria*, *Pseudomonas*, *Staphylococcus*, and *Escherichia*. They also revealed that the copper-NHCs displayed comparable activity to the silver-NHCs, which suggested the copper-NHCs would serve as a potential candidate. However, it still remains challenges whether copper-NHCs would readily effective as cheap alternative for expensive and noble metal silver when encounter oral microbial environment.

Within this strategy, we describe herein the synthesis an array of copper-NHCs, and systematically investigated the relationship of the chemical structure and the performance. All these complexes were screened for antibacterial activity against oral pathogens in vitro. We hypothesized that synthesized complexes could take an effect on oral bacteria, especially for *S.mutans*.

2. Methods

The NMR data of these copper-NHCs compounds were investigated on a Varian Mercury-Plus 400 MHz spectrometer at ambient temperature with the decoupled nucleus, using $CDCl_3$ as solvent and referenced versus TMS as standard.

2.1 The synthesized procedure for copper complexes

All these copper complexes were synthesized according to the following procedures[22]. Among them, the **Cu1** and **Cu3** were the reported compounds[23], while **Cu2**, **Cu4-Cu7** were the firstly reported complexes.

A vial was charged with imidazolium salt (1.0 mmol), CuCl (1.0 mmol) and K_2CO_3 (2.0 mmol). Acetone (1.0 mL) was added into the mixture and stirred at 60 °C for 24 h. When reaching the time, the solid was filtered and washed with dichloromethane. Then the filtrate was concentrated and hexane (3.0 × 2.0 mL) was added. The precipitating solid of desired product was dried under vacuum in the range of 72–90% yields.

Cu2 was obtained in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.30 (m, Ar-H, 8H), 7.27–7.22 (m, Ar-H, 5H), 7.19–7.13 (m, Ar-H, 8H), 7.07 (s, Ar-H, 2H), 6.94 (s, Ar-H, 4H), 5.54 (s, CH, 2H), 2.07 (s, CH_3, 12H).^{13}C NMR (101 MHz, $CDCl_3$) δ 178.9, 145.5, 143.2, 135.8, 134.7, 129.7, 129.5, 128.5, 126.5, 122.2, 56.4, 31.6, 22.6, 17.9, 14.1.

Cu4 was obtained in 90% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.1 Hz, Ar-H, 2H), 7.42 (dd, *J* = 8.3, 7.0 Hz, Ar-H, 2H), 7.10 (s, Ar-H, 4H), 7.05 (d, *J* = 6.7 Hz, Ar-H, 2H), 2.42 (s, 6H), 2.22 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.1, 139.6, 137.9, 134.4, 133.6, 130.6, 129.7, 129.6, 128.2, 127.7, 125.3, 120.9, 21.2, 17.9.

Cu5 was obtained in 87% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, *J* = 8.3 Hz, Ar-H, 2H), 7.45 (dd, *J* = 8.2, 7.1 Hz, Ar-H, 2H), 7.39 (t, *J* = 7.5 Hz, Ar-H, 8H), 7.29 (t, *J* = 7.3 Hz, Ar-H, 4H), 7.24 (d, *J* = 7.2 Hz, Ar-H, 8H), 7.08–6.99 (m, Ar-H, 6H), 5.65 (s, CH, 2H), 2.20 (s, CH3, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 184.0, 145.6, 143.3, 137.9, 134.7, 134.5, 130.6, 130.0, 129.6, 129.5, 128.5, 128.3, 127.7, 126.5, 125.2, 120.9, 56.5, 18.1.

Cu6 was obtained in 82% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.3 Hz, Ar-H, 2H), 7.52 (t, *J* = 7.7 Hz, Ar-H, 2H), 7.46–7.31 (m, Ar-H, 6H), 6.98 (d, *J* = 6.9 Hz, Ar-H, 2H), 2.62 (q, *J* = 7.5 Hz, CH_2, 8H), 1.20 (t, *J* = 7.6 Hz, CH_3, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.1, 140.8, 138.4, 134.9, 130.6, 130.3, 129.7, 128.3, 127.7, 127.4, 125.2, 121.0, 24.6, 15.0.

Cu7 was obtained in 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.2 Hz, Ar-H, 2H), 7.59 (t, *J* = 7.8 Hz, Ar-H, 2H), 7.46–7.39 (m, Ar-H, 6H), 7.01 (d, *J* = 6.9 Hz, Ar-H, 2H), 2.84 (dt, *J* = 13.8, 6.9 Hz, CH, 4H), 1.35 (d, *J* = 6.9 Hz, CH_3, 12H), 1.13

(d, J = 6.9 Hz, CH_3, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.8, 145.6, 138.7, 133.1, 130.8, 129.8, 128.3, 127.8, 125.3, 124.5, 121.0, 28.9, 24.8, 23.7.

2.2. The antimicrobial activity assessment for copper-containing complexes

Before assessing antimicrobial activity, the copper complexes were dissolved in dimethyl sulfoxide(DMSO) at a primary concentration of 10 mg/mL and stored at -20 °C.

2.2.1. Minimum inhibitory concentrations (MIC) of the microorganism

The minimum inhibitory concentrations of complexes against ten microorganism were determined for the complexities by the broth microdilution, according to the guideline of Clinical and Laboratory Standards Institute[24]. *S.mutans* UA159 as the most important causative pathogenic microorganism were first tested, then the drugs screened accordingly were detected for subsequent microorganism. In general, each microorganism was grown overnight in 37 °C to reach the mid-logarithmic phase. Cultures were diluted to 5×10^5 CFU/mL in 96 well flat-bottom plates, whereas fungus to 5×10^3CFU/mL. Final drug concentrations ranging from 500 μg/mL to 0.49 μg/mL were serially diluted in micro-plates with bacterial suspensions by incubating at 37 °C for 24 h. Combine optical densities at 600 nm with naked eye to evaluate the experimental result. Chlorhexidine Gluconate was selected as a positive control. Microorganisms with corresponded concentration of DMSO and Brain heart infusion (BHI, Difco, USA) broth medium served as a negative control and blank meanwhile.

2.2.2. Microorganism strains and growth conditions

A total of ten oral microorganisms were tested in this experiment, including seven Gram-positive bacteria, two Gram-negative bacteria and one fungus. *S.mutans* UA159, *Streptococcus gordonii* ATCC10558 (*S.gordonii*), *Streptococcus sangguis* ATCC10556 (*S.sangguis*), *Enterococcus faecalis* OG1RF (*E.faecalis*), *Lactobacillus casei* ATCC393 (*L.casei*), *Lactobacillus acidophilus* ATCC4356 (*L.acidophilus*), *Actinomyces naeslundii* ATCC19039 (*A.naeslundii*) were grown in Brain heart infusion(BHI), while *Candida albicans* SC5314 (*C.albicans*) cultured in Sabouraud's dextrose broth(SDB, HKM, CHN). Other bacteria including *Fusobacterium nucleatum* ATCC10953 (*F.nucleatum*), *Actinobacillus actinomycetemcomitans* ATCC43717(*A.a*), were grown in BHI supplemented with 0.5%yeast extract (Oxoid, UK), 0.04% L-cysteine(Sigma, USA), 5 μg/mL hemin(Macklin, CHN) and 1 μg/mL vitamin K1(Aladdin, CHN) solution. All bacteria strains except *C.albicans* incubated in an anaerobic chamber(90%N_2,5%H_2 and 5%CO_2,Thermo Scientific, MA, USA) at 37 °C, while *C.albicans* reproduced in aerobic condition with shaking at 200 rpm.

2.2.3. Time-kill assay

To study killing kinetics on live bacteria, complexes effects against *S.mutans* UA159 was determined. Briefly, *S.mutans* were grown previously and diluted to 2×10^7 CFU/mL treated with carbene at different concentrations of 1/2MIC, MIC, 2MIC. Culture without drug was used as control group. After 0, 2, 4, 6, 8,12 and 24 h post-inoculation, cultures were diluted, spread and incubated onto BHI agar plates at 37 °C for 24 h to quantify viable cells numbers.

2.3. Evaluate biofilm formation of *S.mutans*

2.3.1. Assess biomass of *S.mutans* biofilm

Total biomass was investigated using the crystal violet staining. *S.mutans* UA159 were suspended to 2×10^7 CFU/mL in 200 μL BHI liquid medium with 1% sucrose (BHIS).Treated with complexes, bacterial cell suspensions were used to form biofilm at 37 °C in approximately 5% CO_2 for 24 h incubation. After that, washed twice with sterile phosphate-buffered

saline (PBS) and fixed by 100% methyl alcohol for 15 min, then replaced with 200 μL 0.1% crystal violet (CV, Sigma, USA) for 15 minutes. The biofilm washed with PBS to remove the residual dye. The adherent biomass stained with crystal violet at the bottom of plates were released by 200 μL 95% ethanol. The absorbance values were measured at the wavelength of 595 nm using a microplate reader (Infinite 200, Tecan, SUI).

2.3.2. Evaluate metabolic activity of *S.mutans* biofilm

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was utilized to assess the metabolic activities of viable biofilm cells. MTT (Amresco, USA) powder was dissolved in PBS at a working concentration of 5 mg/mL under sterile conditions and stored at -20 °C. Biofilm formed as described above. Each well was added 50 μL MTT solution and incubate at 37 °C for 3 h in the dark. After removing MTT solution, used 100 μL DMSO to dissolve Formazan and then transferred solution to a new plate. Wells contained no bacteria were used as blank controls, while without complexes were served as negative ones. The absorbance of OD_{570nm} values was detected. Metabolic activities = 1-(ODnegative − ODtest)/(ODnegative-OD_{blank}) × 100%.

2.3.3. LIVE/DEAD staining and confocal laser scanning microscope (CLSM)

S.mutans UA159 was treated with complexes to establish biofilm on glass slides as described above. The 24 h biofilm was mildly washed to remove planktic bacteria by PBS and then stained with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, USA) containing SYTO 9 and propidium iodide in dark for 15 minutes. Excess dye was removed by washing the biofilm with PBS. A confocal laser scanning microscope (Zeiss, Germany) equipped with a 63 × oil immersion objective lens was applied to capture images, which then analyzed by COMSTAT(25).

2.3.4. EPS synthesis measurement

S.mutans UA159 inoculated in BHIS as previously described with Alexa Fluor 647 (Invitrogen, USA) labeled dextran conjugate. Similar to procedures described, the biofilm was consequently washed by PBS and stained with SYTO 9 for 15 minutes. The samples were captured with CLSM.

2.3.5. Scanning electron microscopy (SEM)

To observe morphological changes of *S.mutans* UA159 biofilm, treated by complexes, bacteria cells were grown in BHIS and exposed to a screened working concentration for at 37°C. After 24 h, samples were dehydrated in an ethanol series (30, 50, 70, 90, and 100◦GL), critical-point dried with CO_2, coated with gold, and observed under a scanning electron microscope.

2.3.6. Cytotoxicity assay of copper complexes in vitro

Cytotoxicity of complexes were assessed in human gingival epithelial cells(HGECs)using the Cell Counting Kit-8 (CCK-8; Dojindo, Japan). HGECs were purchased from ATCC (Manassas, VA, USA) and cultured in Defined keratinocyte serum free medium (Gibco,USA). Assays were performed in 96-well plate with 10000 cells per well. After incubation in a 5% CO_2 atmosphere for 24 h at 37 °C, the plates were washed with sterile PBS. Then 100 μL of four gradient concentration drugs (0.98 μg/mL, 1.95 μg/mL, 3.91 μg/mL, 7.81 μg/mL,) in cell culture medium were added and continued to incubate for a further 10 minutes or 24 h. The negative control without drugs but with cells and medium, while the blank control was only incubated with cell culture medium. Medium was completely removed and substituted with 100μL of fresh cell culture medium and 10μL CCK-8 reagent. Plates were incubated for another 2 h and then read the absorbance at a wavelength of 450 nm on the microplate reader.

2.4. Statistical analysis

All experiments were repeated three times independently. One-way ANOVA was performed to multiple sets of samples to detect the significant differences, followed by a Dunnett test. Two groups of samples were counted by unpaired *t*-test. Statistical analysis was performed with GraphPad Prism version 7.0 A *P* value of < 0.05 were considered as the significant level.

3. Results

3.1. The characterization of copper-NHCs complexes

The NHCs copper complexes were readily synthesized through the reaction of corresponding imidazolium chloride salt with $CuCl_2$ in the presence of K_2CO_3, which affording the desired copper complexes in high yields (Scheme 1). It is noteworthy that these copper complexes are rather stable toward air and moisture, which were even stable in the DMSO solution for several months and no decomposition was determined. The structures of these complexes were established by the ^1H NMR and ^{13}C NMR (Additional file 1), for which the resonance of Cu – C_{NHC} bond appeared in the range of 178.9-185.8 ppm and the disappearance of the low resonance of the NC*H*N.

3.2. Effective against *S.mutans* and other oral pathogens

In order to preliminarily evaluate the antimicrobial activities of synthetic complexes, we firstly tested the MIC of Copper complexes on *S.mutans* UA159. As shown in Table 1, the **Cu1** complex with 2,6-dimethyl groups on *N*-moieties, showed excellent biological activities at a low value of 1.95 μg/mL, which exhibited comparable activities toward the most commonly applied oral bacteriostatic agent of chlorhexidine. In contrast, the **Cu2** with 2,6-dimethyl groups, while introducing a bulky benzhydryl on para-position of the *N*-moieties, was also selected and moderate efficiency was found (31.30 μg/mL). Moreover, the more lipophilic **Cu3** complex, which is installed with 2,6-diisopropyl groups on *N*-moieties, turned out to be minor activity of more than 500 μg/mL against *S.mutans* UA159. It is noteworthy that **Cu3** was previously displayed excellent performance for the inhibitory activity against *Listeria*, *Pseudomonas*, *Staphylococcus*, and *Escherichia*(26). Subsequently, other copper complexes (**Cu4-Cu7**) with acenaphthyl backbones as well as different *N*-moieties were then screened. As shown in Table 1, it is suggested that rapid decline efficiency was accomplished by the increasement of the steric around the copper complexes.

With the primary results in hand, we selected **Cu1** to determine its time-kill effect on *S.mutans*. According to Fig. 1, the inhibitory behavior obviously started after adding drugs and remained for 24 hours(h) at MIC. The viability of *S.mutans* displayed steady reduction after treated with **Cu1** at MIC. At 2MIC, the bactericidal effect was even more pronounced, with a 99.85% substantial reduction in bacterial cells after 2 h of treatment and complete killed by 4 h.

Table 1
In vitro minimum inhibitory concentrations assessment of synthetic complexes on planktonic *S.mutans* UA159

MIC(μg/mL)	
	S.mutans UA159
Cu1	1.95
Cu2	31.30
Cu3	> 500
Cu4	15.63
Cu5	> 500
Cu6	> 500
Cu7	> 500
chlorhexidine	0.49

Nine other oral pathogens including both Gram-positive, Gram-negative bacteria and fungus were examined immediately after *S.mutans*. We are pleased to discover that **Cu1** was superior to chlorhexidine in inhibiting 6 oral pathogenic bacteria, especially against *A.naeslundi* and *S.gordonii*, with the MIC were 0.49 μg/mL and 0.98 μg/mL, respectively. Meanwhile, the MIC on *C.albicans* SC5314 of **Cu1** was 3.91 μg/mL, which was lower than the MIC 7.81 μg/mL shown by chlorhexidine (Table 2) .Quite intriguingly, although showing broad-spectrum antibacterial activity against both Gram-positive and Gram-positive bacteria, it is also observed that **Cu1** against Gram-positive bacteria was slightly better than Gram-negative ones. **Cu1** showed prominent performance in against *A.naeslundi*, *S.gordonii*, *S.sanguis*, *S.mutans* and *E.faecalis* (MIC 0.49 to 3.91 μg/mL), which were all Gram-positive pathogens. However, MIC of two Gram-negative ones including *A.a* and *F.mucleatum* was 1.95 and 31.25 μg/mL.

Table 2
Antibacterial activities of **Cu1** and chlorhexidine on multiple oral pathogens

MIC(μg/mL)									
	A. naeslundi *	*S. gordonii* *	*S. sanguis* *	*A. a* **	*E. faecalis* *	*C. albicans* ***	*L. casei* *	*F. nucleatum* **	*L. acidophilus* *
Cu1	0.49	0.98	1.95	1.95	3.91	3.91	15.63	31.25	31.25
chlorhexidine	0.98	1.95	3.91	3.91	7.81	7.81	31.25	7.81	15.63
*: Gram-positive bacteria; **:Gram-negative bacteria; ***:fungus									

Combining Table 1 and Table 2, **Cu1** have shown stronger inhibited potency among the 7 synthesized complexes. Notably, **Cu1** exhibited better activities against multiple bacterial strains in comparison with chlorhexidine. Inspired by the excellent biological performance of **Cu1**, further investigation on the formation capacity of *S.mutans* UA159 biofilm treated with this copper(I)-containing Complexes.

3.3. Complexes inhibited *S.mutans* UA159 biofilm formation

CV assays were conducted to evaluate *S.mutans* biofilm formation under the influence of complexes. Clearly, there were obvious distinction in overall biomass compared with the drug-free group($P < 0.001$). In the presence of **Cu1**, the biofilm biomass presented no significant changes at 1/2 MIC, but it decreased sharply to nearly 2.45% at MIC and could not be detected in concentrations corresponding to 2MIC(Fig. 2a).

MTT assays were used for metabolic activity of biofilm at the same concentration of **Cu1** as mentioned above. These results of biofilm metabolism were consistent with the results of CV experiment ($P < 0.001$). Compared to control group, biofilm metabolic activity declined to 16.8% at MIC treated with **Cu1**(Fig. 2b).

3.4. Complexes changed biofilm structure and morphology of *S.mutans*

The biofilm of *S.mutans* treated with **Cu1** at MIC for 24 h were investigated by a confocal laser scanning microscope(Fig. 3). In presence of **Cu1**, the microarchitecture of biofilm demonstrated visibly sparse and loose distribution compared with that of the negative control, which consequently resulted in apparent reduction in the total biofilm biomass ($P < 0.001$) and EPS synthesis ($P < 0.01$).

Consistent with CLSM assay, there was no obvious biofilm formation observed compared with the free-drug group under the scanning electron microscope (Fig. 4). Addition of **Cu1** resulted in the atypical morphology of *S.mutans* with irregular edge and significant reduction of extracellular polysaccharides. Furthermore, the remaining bacterial cells also had difficulty forming bacterial aggregates and biofilm microarchitecture.

3.5. Complexes exhibited certain cytotoxicity on HGECs

Next, we examined the cytotoxicity of **Cu1** on HGECs in order to investigate its biocompatibility (Fig. 5). Clearly, **Cu1** exhibited no significant inhibitory effects in a short period of time (10minutes) below 1.95 μg/mL (MIC) ($p > 0.05$). Although the drug's toxicity gradually increased with the augmentation of concentration and the extension of time (24 h), it is noticeable that Cu1 exhibited an effect on approximately 50% viability of HGECs at the MIC. ($p < 0.001$).

4. Discussion

Copper, as a crucial component for animals and microorganisms, has been used as antifungal or antibacterial agent for a long time[27, 28]. It has been proved that certain concentration of copper ions can poison bacterial cells by inactivating key metal enzymes, especially those containing soluble iron and zinc[29–31]. By chelating small molecular ligands, the antibacterial properties of copper can be selectively adjusted, and even achieve the synergistic effect of high-level antibacterial[27]. In the past decade, the biological activity of metal NHCs have been received much attention because of their strong σ-donor capacity that make the high stability of the transition metal complexes[32–36]. Therefore, we tested and reported a series of copper-NHCs presenting effective inhibitory activity to oral pathogens, especially for free-living *S.mutans* and its biofilm form.

In the present study, it is highlighted that the much less lipophilic **Cu1** exhibited strong inhibitory potency against multiple oral planktonic bacteria and investigated the effect for biofilm formation. Comparatively, the bulkier steric NHCs copper complexes of **Cu2-Cu7**, all afforded much less efficiency. In addition to exploring the minimum inhibition concentration, time-kill assay further confirmed that **Cu1** was bactericidal and its sustained inhibition and killing effect at MIC, as well as the short-term and efficient eradication at twice this concentration. Meanwhile, **Cu1** acted a pivotal part in disrupting biofilm formation, even on a relatively high density of bacteria (2×10^7 CFU/mL), which was verified by a series of subsequent experiments. Accordingly, it is very reasonable to speculate that the capacity of **Cu1** to affect biofilm formation is based on the killing effect on bacterial cells.

CV and MTT assays showed effective reduction of **Cu1** on the biofilm biomass and metabolic activities of biofilm at MIC. Combined with the results of previous section, our conjecture was confirmed. This biofilm inhibition is most likely due to the reduction in the number of bacteria. Biofilm morphology observation experiments also confirmed this phenomenon.

EPS production, which directly mediates microbial adherence to a surface, forms a polymeric matrix that enhance mechanical stability of biofilm[7]. Hence, EPS is recognized as one of key virulence factors of oral biofilm in terms of caries development[37, 38]. Both the CLSM and the SEM were used to assess the biofilm microstructure, the EPS production was strikingly reduced. This greatly contributed to prevent the formation of a three-dimensional structure of the biofilm. At the same time, microscopic studies displayed **Cu1** causes altered phenotype of the biofilm, with reduced number. SEM also displayed deformed, distorted, and collapsed cells, while normal shape is short rod. The altered cell morphology appears to be one of the major causes of deficient biofilm. Although the specific mechanism of action still needs further study, it has been widely studied that organic ligands combing with copper modulate activity by neutralizing the electric charge of the copper ion, increasing the lipophilicity of the complex promoting transport through cell membrane, or intercalating to DNA or interacting non-covalently with proteins[39].

The biocompatibility of synthesized copper-NHCs complexes is the impediment for its clinical application. These results demonstrated that short-duration treatment to **Cu1** below 1.95 μg/mL (MIC) has no negative impact to human oral cells, while long-time and high-dose use can affect cell activity. Actually, it has been known that when **Cu1** used as an mouthwash agent at this concentration for a short period of time, it can already play a role in affecting reproduction of *A.naeslundi*, *S.gordonii*, *S.sanguis*, *A.a* and *S.mutans*. **Cu1** has the characteristics of high activity and low cytotoxicity in a short-term treatment mode, making it a preferable agent for preventing dental caries. Therefore, these Copper-containing complexes have been discovered as potential oral hygiene products.

5. Conclusions

In summary, a type of well-defined and air-stable copper-containing small molecule complexes were synthesized and characterized. Their inhibition activities of (**Cu1-Cu7**) was screened. It was found that **Cu1** provided significant excellent performances, which suggested that the less lipophilic and less steric copper complexes would be effective toward the bacterial. **Cu1** also impeded *S.mutans* biofilm formation and EPS synthesis. This could open promising perspectives for exploration and development of novel metal complexes, which were applied for biofilm control associated to oral pathogens.

Abbreviations

S.mutans
Streptococcus mutans
EPS
Extracellular polysaccharides
NHCs
N-heterocyclic carbenes
DMSO
Dimethyl sulfoxide
MIC
Minimum inhibitory concentrations
S.gordonii
Streptococcus gordonii
S.sangguis
Streptococcus sanguis

E.faecalis
Enterococcus faecalis
L.casei
Lactobacillus casei
L.acidophilus
Lactobacillus acidophilus
A.naeslundii
Actinomyces naeslundii
F.nucleatum
Fusobacterium nucleatum
A.a
Actinobacillus actinomycetemcomitans
BHI
Brain heart infusion
BHIS
Brain heart infusion with sucrose
PBS
Phosphate-buffered saline
SEM
Scanning electron microscopy
CLSM
Confocal laser scanning microscopy
HGECs
Human gingival epithelial cell
CCK-8
Cell Counting Kit-8

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

TP ,FL and YZ designed the study. TP performed the experiments and collected the data. TP, YW analysed the data. TP and FL wrote the manuscript. HL and YZ revised the manuscript. All authors have read and approved the final manuscript.

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Figures

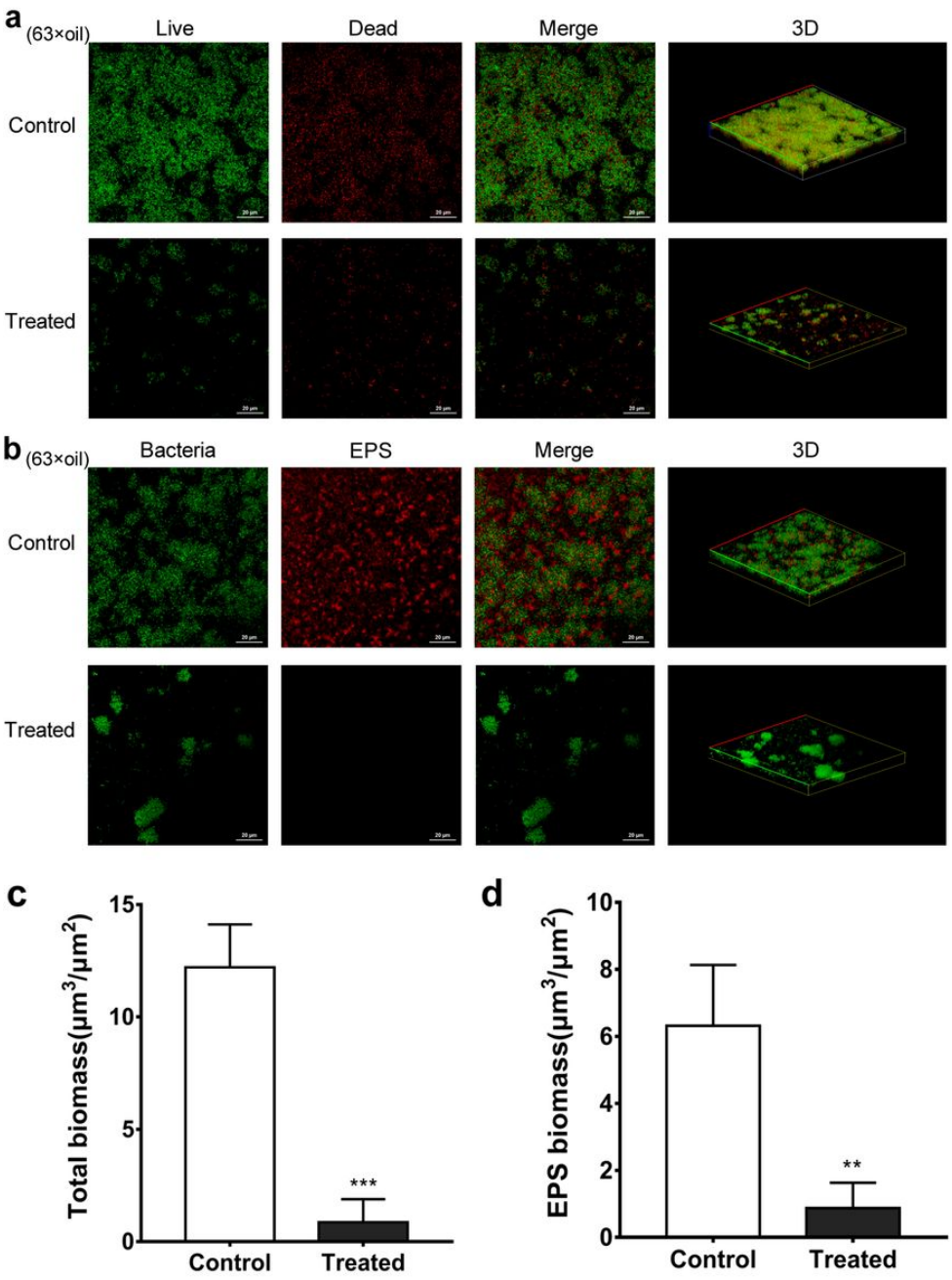

Figure 1

S.mutanstime-killcurve The time-kill kinetics of Cu1 against S. mutans UA159. The bacteria were treated at the concentration of 1/2, 1, 2MIC. The pathogen untreated with compounds were selected as control. The data were presented as mean ± SD.

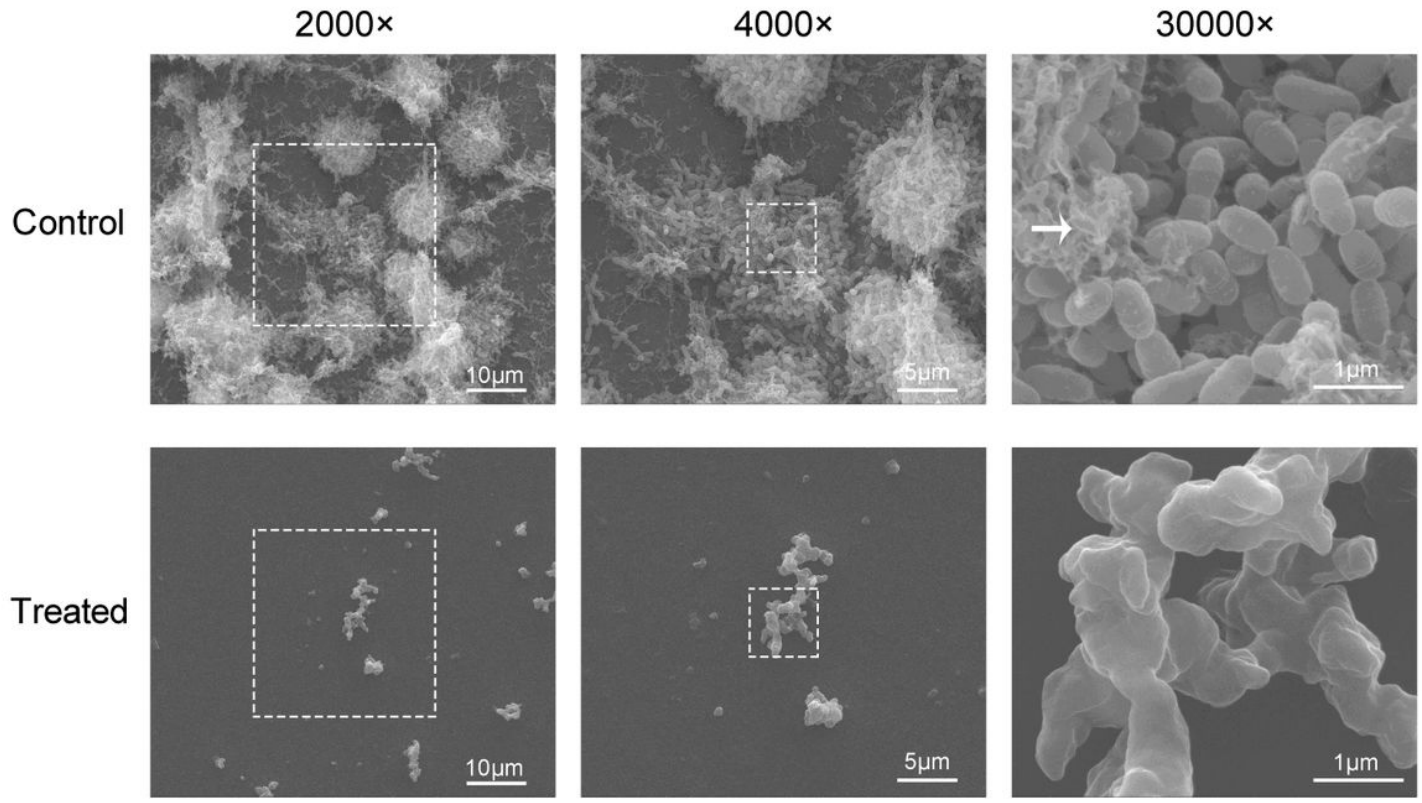

Figure 2

CV and MTT assays The CV (a) and MTT(b) assay of Cu1 against S. mutans UA159 biofilm formation at 24 h. The data were presented as means ± SD. ***P< 0.001, significant difference from the control group.

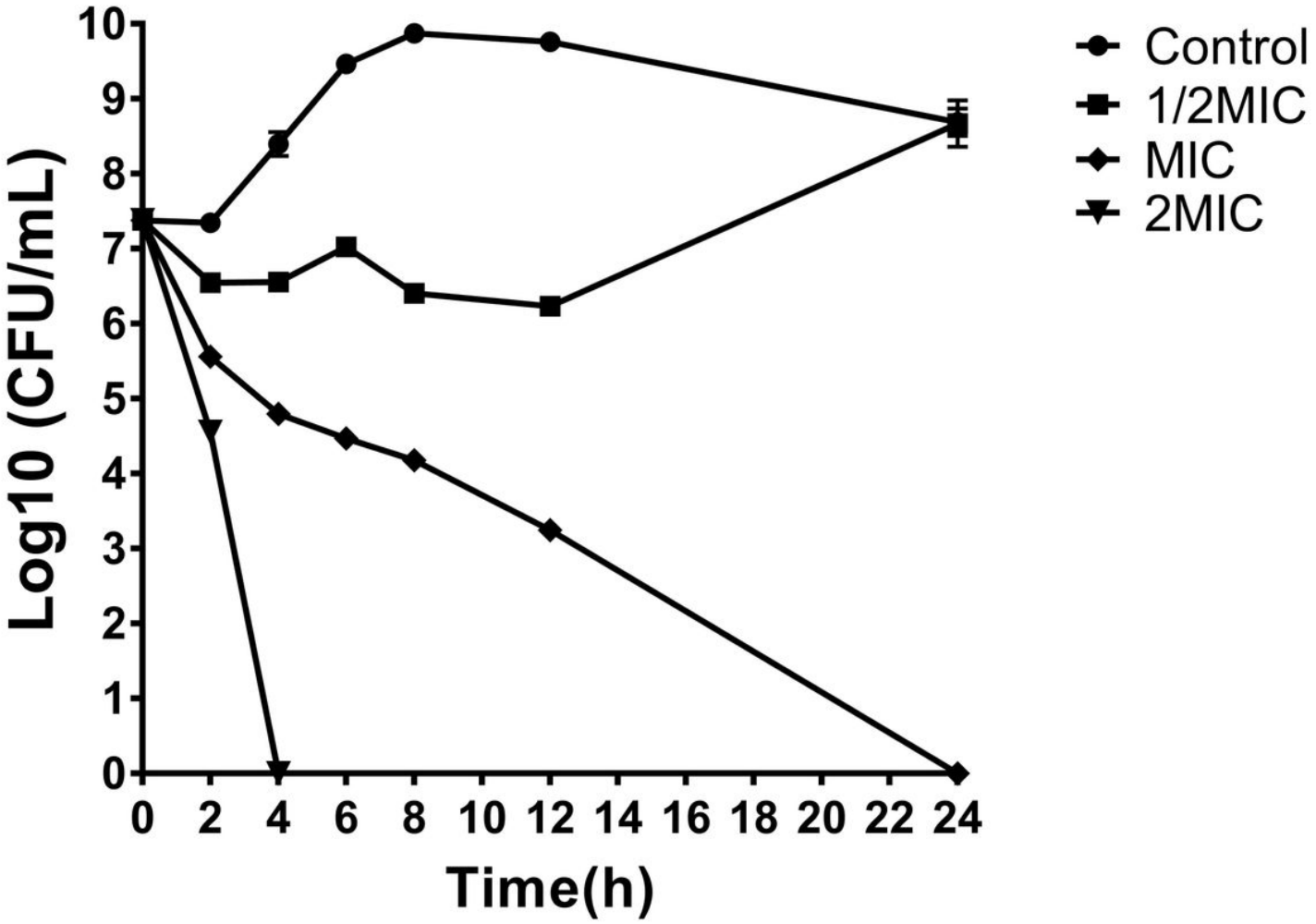

Figure 3

Live/dead and EPS staining by CLSM CLSM images of S.mutans biofilm. Single, double channel as well as three-dimensional reconstructions of S.mutans biofilm of control and treated groups. (a) Color green reflected live bacteria; red reflected dead bacteria. (b) Color green indicated microorganism; red indicated EPS. (c) Quantitative analysis of total bacterial biomass in (a). (d) Quantitative analysis of EPS biomass in (b). The data were presented as means ± SD. **P<0.01; ***P < 0.001.

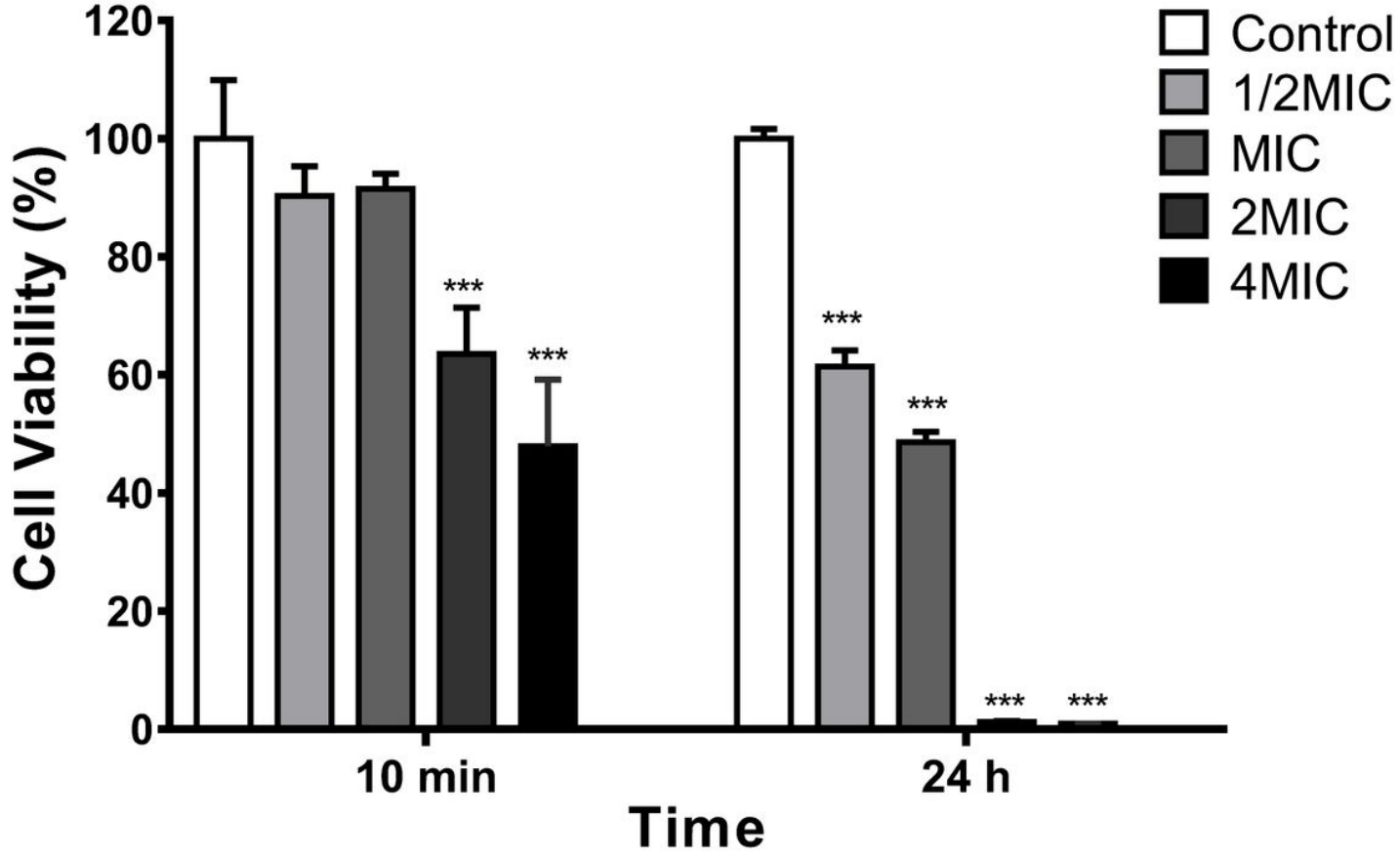

Figure 4

The morphological change of S.mutans The morphological change of S.mutans biofilm with or without Cu1 complexes treatment .Each visual field was in 2000×, 4000× and 30000× magnification. The boxes show enlarged area. The white arrow indicates the EPS in the biofilm. The black arrows indicate the different morphology of S.mutans.

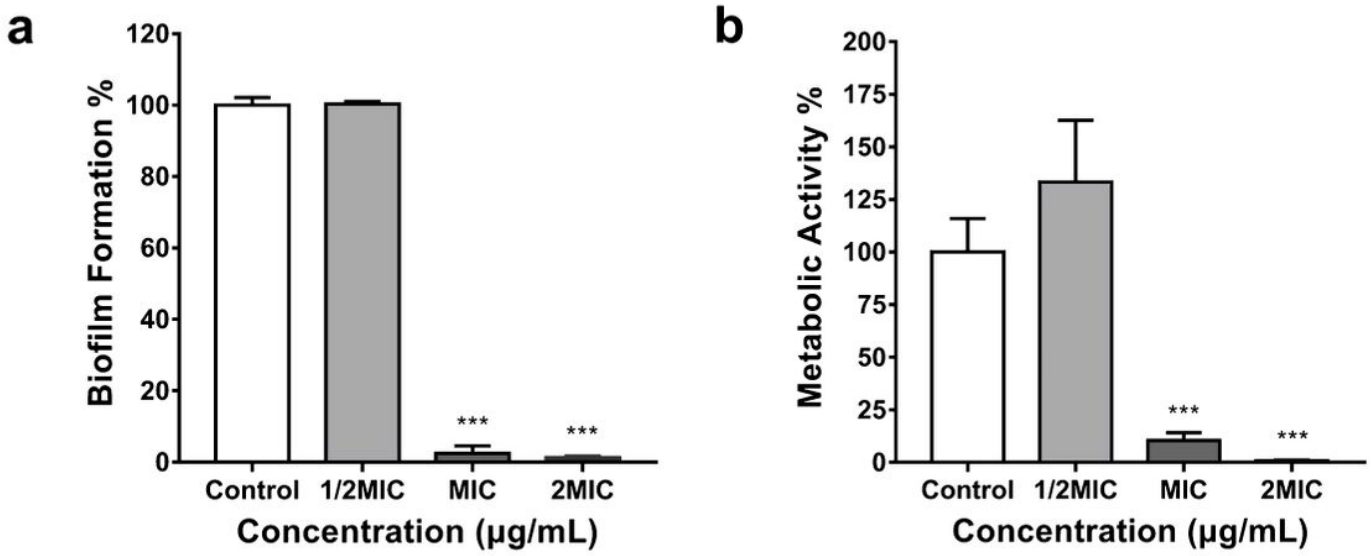

Figure 5

Cytotoxicity of Cu1 on HGECs Cytotoxicity of Cu1(0.49µg/mL to 7.81µg/mL) on human gingival epithelial cells (HGECs) after 10 minutes and 24 h incubation. The data were shown as means ± SD. The asterisks (*) indicate the significant differences (*** P< 0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.jpg
- Additionalfile1.pdf