A Nanostructured Cu(II) Coordination Polymer Based on Alanine as a Trifunctional Mimic Enzyme and Efficient Composite in the Detection of Sphingobacteria

1. Introduction

Pathogenic intracellular bacteria have been proven to trigger numerous chronic or recurrent infectious diseases that pose substantial global public health threats. The main medical route for treating bacteria is antibiotics, but these microorganisms use many mechanisms to survive by becoming resistant [1]. For this reason, the discovery and development of alternative antimicrobial strategies are also critical [2–6]. Additionally, in the last decades, researchers have tried intensively to find new compounds capable of behaving as artificial enzymes, copying the functions of natural enzymes [7]. These artificial enzymes will be able to produce essential chemicals on an industrial scale with a performance that rivals their natural counterparts. Moreover, if we are inspired by the use that the human body makes of enzymes to improve its defense mechanisms, see, for example, the xanthine oxidase that can generate superoxide anion (O₂⁻) and H₂O₂ in the presence of O₂, or the myeloperoxidase that can catalyze the conversion of H₂O₂ into highly reactive oxygen species (ROS) that behave as natural antimicrobials, then artificial enzymes could also be used as alternative...
compounds in the fight against bacteria [8]. Investigations into these two subjects cover an immense variety of materials [9, 10]. However, coordination polymers can be a possible solution to both aspects since they have fascinating characteristics and properties to achieve these objectives. From the chemical point of view, a recently published precedent shows how coordination polymers (CPs) can be highly interesting in manufacturing mimic enzymes with, at the same time, antimicrobial capacity as they can generate ROS in a controlled way [11]. Indeed, one exciting advantage of the CPs as possible artificial enzymes is their facile preparation, nanoprocessability, low cost, and superior stability [12]. Other advantages of the CPs are their tuneability, since the proper selection of the building blocks (metal ions and organic ligand/s) should allow the obtaining of a material with the desired properties [13–16]. Thus, the use of copper (II) as a metal center enables the creation of compounds with different antibacterial mechanisms [17–19]. One can be related to the metal ion release in a controlled manner, favoring obtaining compounds that improve wound healing without generating toxicity. The metal ion release is related to the electrostatic attraction between the positively charged Cu(II) or Cu(I) ions and microorganisms negatively charged cell membranes, resulting in bacterial cell death by damaging the cell wall and plasma membrane [17]. Indeed, considering this type of strategy, A. Lauf et al. have proved that the copper (II) release rate is crucial in antibacterial effectiveness, finding that this efficacy can be improved as the particle size is reduced [20, 21]. Indeed, considering particle insertion into bacteria as a possible antibacterial mechanism, it has been demonstrated that Cu(II) coordination polymer nanofibers are more effective than the same compound in the form of microcrystals [17]. These research studies show that the synthesis of new Cu(II)-based structures with control in the Cu (II) ion release rate is essential [11, 17, 19, 22]. Additionally, the presence of this ion is critical to provide peroxidase [23], catalase, or superoxodismutase (SOD)-like activity. Organic ligands are also crucial to obtaining compounds of biological interest that when prepared with the appropriate synthetic conditions can generate metal-organic gels (MOGs) with three-dimensional network structures capable of harboring solvents. These new phases have been highly interesting for real industrial applications since they facilitate the localized superficial administration of metal ions as Cu (II) in the antibacterial treatment of external wounds [24, 25]. Other studies show that creating new composite materials by a combination of Cu (II) CPs and organic matrices such as polyacrylamide, cellulose, fiber, gelatin [26], chitosan (CS), or cotton [27–30], among others [31], provides improvements in antibacterial effectiveness in addition to new mechanical properties allowing their manufacture. Despite these important discoveries, there are just a few examples of CPs combining multiple enzymatic actions, although none acting as a trifunctional mimic enzyme [32–34]. Here we present a study that combines Cu (II) with a pseudoamino acid (H₂IBA = isophthaloyl bis β-alanine). The synthesis in a single step and sustainable conditions (water and 25°C) allows obtaining a 2D CP that can be nanoprocessed and gelled by sonication of different Cu (II) metal salts. The obtained CP, with the chemical formula [Cu₂(IBA)₂(OH₂)₄]ₙ·6nH₂O shows slow Cu (II) release and is the first example of a trimimic artificial enzyme having peroxidase, catalase, and superoxodismutase like activity, being effective for the resistant Sphingobacterium. In addition, the easy preparation of the corresponding metal-organic gel and also a composite material based on gelatin and the nanocoordination polymer (NCP) will allow the creation of easy-handling and low-cost commercial kits valid for detecting Sphingobacterium [35, 36].

2. Results and Discussion

2.1. Chemical and Morphological Characteristics of 1n. An essential advantage in the synthesis of this CP is the possibility of obtaining it in different phases and sizes by slightly modifying the synthetic conditions. That is, the direct reaction between H₂IBA deprotonated with NaOH and Cu(II) can generate compound 1 as single microcrystals, as polycrystalline, as a colloid formed by nanofibers (1n), or as metal-organic gel (1n@MOG) depending on the starting copper (II) salt (Cu(NO₃)₂ or CuSO₄) and the application of different sonication times, as shown in Figure 1 (methods described in Supplementary Materials) [37]. The characterization of 1, 1n, and 1n@gelatin by IR (Figure S1), PXRD (Figures S2 and S3), and SEM (Figure S4) has been carried out. Structurally speaking, compound 1 is a 2D coordination polymer that crystallizes in the monoclinic Pn space group [38]. Its structure has been described previously by S. Lymeropoulou et al. which consists of Cu(II) dimers where both metal ions present a distorted square pyramidal coordination geometry (Figure 2). The basal plane of both Cu (II) ions is formed by two water molecules (with Cu-O bond distances in the range 1.934–1.983 Å) and by two trans carboxylate oxygen atoms from two different IBA²⁻ ligands: a terminal one, with Cu-O bond distances of 1.926(3) and 1.922(3) Å, and a bridging one, with Cu-O bond distances of 1.961(3) and 1.942(3) Å, for Cu₁ and Cu₂, respectively. The apical position is occupied by the bridging carboxylate oxygen atom of the basal plane of the other Cu (II) ion, giving rise to a central Cu₂O₄Cu dimer. The apical Cu-O bond distances (2.366(3) and 2.402(3) Å) are much longer than the basal ones.

As ultrasounds are used to assist sol-gel transitions [39, 40], a bottom-up approach using sonication and CuSO₄ instead of Cu(NO₃)₂ results in the formation of a metal-organic gel (1n@MOG) which is transformed into a colloid formed by the corresponding nanocrystals (1n) with longer sonication times (Figure 1 (c) and (d)). As shown in Figure S4, the use of CuSO₄ instead of Cu(NO₃)₂ seems to enhance the reduction of particle size. The morphology and dimensions of both 1 and 1n were studied by SEM and AFM (Figures 3 and 4). The average width for 1 ribbons and 1n nanoribbons is 354 ± 170 nm and 170 ± 60 nm, respectively, while the average height for 1n is 17 ± 10 nm. Compound 1n is stable at physiological pHs (between pHs 3 and 8) for long periods of time (one year) (Figures S6
**Figure 1:** Different conditions to obtain compound 1 as bulk material (a), fibers, 1-fibers (b), metal-organic gel, 1n@MOG (c), or nanocrystals, 1n (d).

**Figure 2:** Isoththaloyl bis β-alanine (H₂IBA) coordination modes to Cu (II) metal centers in compound 1 (a). Structure fragment of compound 1 (b). Color code: Cu = light blue, O = red, N = purple, C = grey, and H = white.

**Figure 3:** Continued.
and S7). Additionally, Figure S8 shows thermal stability up to 254°C. It loses all the solvation water molecules at this temperature, and the coordinated ligands decompose mainly into CO2. At temperatures above 100°C, the blue color of compound 1n reversibly transforms into a new less crystalline green color phase due to the loss of the first solvated water molecules (Figures S9–S11). The magnetic properties of 1n show the presence of a metamagnetic behavior with a high critical field of ca. 5.5T at 2K, i.e., the Cu (II) dimer shows a weak antiferromagnetic coupling of \(-6.0 \text{ cm}^{-1}\) that becomes ferromagnetic for applied magnetic fields above 5.5T, as depicted in Figures S12 and S13.

2.2. Catalase Mimicking Activity of 1n. Catalase is responsible for the catalytic decomposition of hydrogen peroxide through its disproportionation reaction into nontoxic dioxygen and water. The catalytic activity of 1n (methods in Supplementary Materials) [37] towards the decomposition of hydrogen peroxide has been investigated in water at 25°C. Initially, the suspension of 1n is dark blue, but after adding 30% (v/v) hydrogen peroxide, it became faded yellow and rapid evolution of gas was observed, such as in Figures 5(a)–5(d). It can be easily understood that the evolved gas is oxygen, and it comes from the catalytic decomposition of hydrogen peroxide solution [41].

Moreover, to quantitatively estimate the catalase activity of compound 1n, a colorimetric assay was performed by titration of excess unreacted H2O2. In this method, the decomposition of H2O2 is estimated spectrophotometrically by a reaction with potassium dichromate/acetic acid reagent [42] (catalytic activity: methods in the Supplementary Materials). In the presence of H2O2, potassium dichromate in acetic acid is reduced to green-colored chromic acetate, which can be measured colorimetrically at 570nm. As shown in Figure 5(e), when H2O2 is added to the 1n, the absorbance signal decreases significantly from 1 to 0.43 after 2.5 hours of incubation. This result demonstrated that compound 1n presents catalase-like activity, directly proportional to the dissociation rate of the H2O2 produced in the samples.

2.3. Peroxidase Mimicking Activity of 1n. The excellent intrinsic peroxidase-like activity of 1n was evaluated by oxidizing chromogenic peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) to form a blue-colored product under the
assistance of H$_2$O$_2$ (catalytic activity: methods in Supplementary Materials) [37]. TMB itself is colourless and displays no absorbance (Figure 6) [43]. As depicted in Figure 6(a), a strong absorption was observed in the group of TMB + 1n + H$_2$O$_2$, indicating that 1n possesses an excellent catalytic activity. Moreover, a time-dependent change in the TMB absorbance was recorded by a UV–vis spectrophotometer at 440 nm (Figure 6(b)).

2.4. Superoxo-Dismutase (SOD) Mimicking Activity of 1n.
To determine if 1n can be a candidate as a mimic antioxidant enzyme useful, for example, in the treatment of disorders related to oxidative stress, we have studied its activity as a superoxodismutase (SOD) [34]. To evaluate this SOD-like activity, an assay with nitroblue tetrazolium (NBT) was performed to quantify the 1n enzymatic activity (catalytic activity: methods in the Supplementary Materials; Figures S14 and S15) [37]. The reaction kinetics between NBT and the xanthine/xanthine oxidase (x/xo) system were measured to establish when the absorbance reaches its maximum value at a wavelength of 560 nm. Triplicate NBT spectra were then performed with different concentrations of 1n in a stepwise manner (0, 0.005, 0.5, 1, 3, and 5 mg/mL), observing a continuous decrease of the signal as the concentration of 1n increases, which disappears entirely at a concentration of 3 mg/mL. Nearly 80% of the superoxides were removed from a concentration of 5 mg/mL of 1n, and IC$_{50}$ = 1.1 mg/mL was calculated (Figure 7) to establish the concentration of 1n that inhibits the rate of NBT reduction by 50%.

2.5. Antibacterial Assays. To determine the copper concentrations produced when compound 1n is in suspension, release assays of the compound in deionized water were carried out (Figure S16). Considering the system’s slow Cu(II) release (45 ppm at 24 h), its nanometric dimensions, synthesis in the water at room temperature in just one step, and its ability to act as a multiartificial enzyme [11], its antimicrobial activity [44] was also explored.

The antibacterial activity of 1n compound and 1n + H$_2$O$_2$ was tested by agar diffusion against different microorganisms (antibacterial experiments in Supplementary Materials) [37] including Gram-positive (Bacillus, Deinococcus, and Lactococcus lactis) and Gram-negative (Escherichia, Pseudomonas, and Sphingobacterium, Alcaligenes). Furthermore, different concentrations of H$_2$O$_2$ alone were checked against Sphingobacterium to discard the growth inhibition by oxygen peroxide’s effect at the range of work concentrations, only observing a slight halo at 300 mM, as shown in Figure S17.

As well, the minimal inhibitory concentration (MIC) and growth curves of the compound 1n without and with H$_2$O$_2$ versus different strains were assayed (Table S1 and Figure S18).

Figure 5: Oxygen bubbles generation when 1n is exposed to H$_2$O$_2$ 30% for (a) $t$ = 0, (b) $t$ = 10 s, (c) $t$ = 20 s, and (d) $t$ = 30 s. Absorbance of chromic acetate in the presence of H$_2$O$_2$ (left bar) and after the incubation with 1n (right bar). The data correspond to mean ± S.D. values from four experiments (e). Statistical analysis was performed using one-way ANOVA Tukey’s test (each group vs. control). *** $P < 0.0001$. 
The results obtained indicate that compound 1n did not show growth inhibition against any strain, which means that 1n is not an antimicrobial agent.

But 1n + H2O2 inhibits the growth of A. faecalis, B. cereus (to a lesser extend), and Sphingobacterium, probably due to the generation of reactive oxygen species [45–47] (Figure 8).

Most isolates from humans are Sphingobacterium spiritivorum which are generally resistant to kanamycin and ampicillin and are susceptible to the quinolones and trimethoprim-sulfamethoxazole.

Bacterial growth of Sphingobacteria was also measured by the optical density method at 600 nm (OD600) overnight at 37°C. After 24 h in the presence of 1n + H2O2, no bacterial growth was detected that performed as an antimicrobial agent (Table 1).

Figure 7: Decrease in NBT absorbance versus progressive increase in 1n concentration (0–5 mg/mL) (a) and 1n concentration that inhibits the NBT reduction rate by 50% (b). IC50 calculation using linear regression analysis, employing five different concentrations of 1n. The data correspond to mean ± S.D. values from three experiments. Statistical analysis was performed using one-way ANOVA Tukey’s test (each group vs. Control). ***P < 0.0001.

2.6. Efficient Composite (1n@Gelatin) in the Detection of Sphingobacteria. The prepared metal-organic gel of 1n (1n@MOG) was not stable at physiological pH (only around pH
Figure 8: Antibacterial experiments photographs of compound $\text{In} + \text{H}_2\text{O}_2$ (25 mM) where $\text{CuIBO}_3 = \text{In}$; $\text{IBO}_3 = \text{H}_2\text{IBA}$; $\text{Amp} = \text{ampicillin}$) tested on $\text{Sphingobacterium}$ (a), $\text{Alcaligenes faecalis}$ (b), and $\text{Bacillus cereus}$ (c). Ampicillin is used as antibiotic control.

Table 1: OD values at 600 nm of the $\text{Sphingobacterium}$ in the presence of kanamycin antibiotic, $\text{H}_2\text{O}_2$, $\text{In}$, and $\text{In}$ plus $\text{H}_2\text{O}_2$.

<table>
<thead>
<tr>
<th>Strain/compound</th>
<th>OD$_{600\text{nm}}$ 24h 37°C</th>
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<tbody>
<tr>
<td>$\text{Sphingobacterium} + \text{In}$</td>
<td>2.67</td>
</tr>
<tr>
<td>$\text{Sphingobacterium} + \text{Kanamycin}$</td>
<td>2.44</td>
</tr>
<tr>
<td>$\text{Sphingobacterium} + \text{H}_2\text{O}_2$</td>
<td>2.34</td>
</tr>
<tr>
<td>$\text{Sphingobacterium} + \text{CuSO}_4$</td>
<td>1.35</td>
</tr>
<tr>
<td>$\text{Sphingobacterium} + \text{In} + \text{H}_2\text{O}_2$</td>
<td>0.05</td>
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Figure 9: FESEM images of $\text{Sphingobacterium}$ with no treatment (a) and after being treated with 100 $\mu$L $\text{In} + 50 \mu$L $\text{H}_2\text{O}_2$ (30%) for 24 h(b).

Figure 10: Photographs of $\text{In@Gelatin}$ (a) and $\text{Sphingobacterium spiritivorum}$ colony count method (50 $\mu$L of overnight of culture after $10^8$ dilution) (b) where 490 colonies were counted for the blank experiment (40 $\mu$L of $\text{H}_2\text{O}_2$ at 25 mM) and (c) 210 colonies were counted after being treated $\text{Sphingobacterium}$ with $\text{In@Gelatin}$ (40 $\mu$L at 3 mg/mL) plus $\text{H}_2\text{O}_2$ (40 $\mu$L at 25 mM).
3). Therefore, we have used gelatin as a biocompatible organic matrix in order to process 1n in a moldable and easy way creating a gel-like composite (Figure 10(a)). This new composite allows the release and diffusion of 1n during the incubation stage thanks to its excellent solubility in water at 37°C. With compound 1n in this format, we have achieved an improved manufacture and handling of the material, facilitating its application in vitro tests compared to bulk material.

The antibacterial efficacy of 1n@Gelatin was checked against *Sphingobacterium* by the colony count method (antibacterial experiments in Supplementary Materials) [37]. The number of grown colonies of *Sphingobacterium* over-night at 37°C was 490 colonies, while in the presence of 1n@Gelatin plus H2O2 (25 mM) it was half (Figure 10(b)). From these results, the percentage of inhibition (K inhibition (%) of 1n@Gelatin was evaluated versus *Sphingobacterium* and *E. coli* DH5α. The results showed K = 57% for *Sphingobacterium* inhibition, while there was no decrease in the number of colonies for *E. coli* DH5α.

### 3. Conclusions

This research raises the potential use of coordination polymers as new useful materials in two significant fields of research, allowing the obtaining of new artificial enzymes with the capacity of inhibiting the growth of bacteria resistant to the usual antibiotics. The adequate selection of the ligands has enabled the design of the first artificial trienzyme model (peroxidase, catalase, and superoxodismutase) based on a Cu (II) CP with modified alanine. It is worth emphasizing that studies based on the search for enzyme mimics are focused on organic materials, but the study of artificial enzymes with CPs is practically nonexistent, despite the good characteristics that are present in CPs based on biologically relevant ligands. Remarkably, its excellent catalytic activity in the presence of minimal amounts of hydrogen peroxide is essential for its antibacterial effect on three resistant bacteria strains. Another critical aspect of this work is that Gram negative bacteria (*Alcaligenes faecalis* and *Sphingobacterium*) are usually involved in respiratory infection processes and present a vast antibiotic resistance, making this CP a powerful alternative for its use against these types of pathogens. The damages caused to the bacterial wall indicate indirect evidence of the generation of oxidizing hydroxyl species or reactive oxygen species due to the CP plus minimal concentrations of H2O2. Finally, the transformation of the CP into a metal-organic gel (MOG) could facilitate its use in the manufacture of new commercial *Sphingobacterium* detection kits. However, the MOG obtained does not present enough stability at physiological pH. For this reason, as a proof of concept, this compound has been homogeneously dispersed in soluble gelatin as an easily useable and moldable organic matrix with excellent antibacterial results which will allow the manufacture of low-cost, comfortable, easy-to-use, and affordable commercial *Sphingobacterium* kits for assessing the presence of microorganisms in a test sample employing the identification of the inhibition of the microorganism.

### Data Availability

The data underlying the findings of this paper are publicly available. All the obtained and used data are available and have been deposited in a public repository whose access link is: https://doi.org/10.21950/NR3KKL. Additionally, they are included in the article in the form of supplementary material (PDF file with the name: SI_bioinorg_chem_app_20_1_22).

### Conflicts of Interest

The authors declare that there are no conflicts of interest.

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### Supplementary Materials

S1 experimental section: materials and instrumentation; S2 synthesis of 1, 1n, and 1n@Gelatin; S3 characterization of 1, 1n, and 1n@Gelatin; S4 solvent and thermal stability of 1n; S5 study of magnetic properties; S6 catalytic activity: methods; S7 copper release and antibacterial experiments; and S8 references. This material can be found in https://edatos.consortiomadrono.es/dataset.xhtml?persistentId=doi:10.21950/NR3KKL. (Supplementary Materials)

### References


[10] H. Wei and E. Wang, "Nanomaterials with enzyme-like characteristics (nanozymes): next-generation artificial en-


