

Evaluating the Copper-binding Properties of the Antifungal Peptide Histatin 5

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Thesis submitted in partial fulfillment of  
the requirements for the degree of Master of Science in the Department of  
Chemistry in the Graduate School  
of Duke University

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ABSTRACT

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## Abstract

Histatins are a family of histidine-rich peptides that are found in saliva. This family of peptides has antifungal properties. In this family, histatin 5 is the most efficient antifungal peptide against *Candida albicans*. The mechanism for the antifungal activity of histatin 5 is still unknown. Previous studies suggest that the metal-binding properties of histatin 5 may have some connection to its antifungal activity. Although there is some evidence that histatin 5 can bind Cu(II), currently there is no conclusive data on the Cu(I) binding properties of histatin 5. This work focuses on investigating the copper-binding properties of histatin 5 and analyzing the role these copper-binding properties may play in the antifungal activity of histatin 5. Model peptides of histatin 5 have been synthesized and copper binding studies were performed by UV-Vis spectroscopy and mass spectrometry. Reactive oxygen species formation process was studied by fluorescence assay.

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# 1. Introduction

## 1.1 Fungal infection and antifungal drugs

Fungal infections have become a threat to human life and the occurrence of these infections has been increased over the years.[1] Fungal infections can occur in any part of human body including eyes, nails, skin, alimentary tract, or can be systemic. [1, 2] Anyone can suffer from fungal infections, however, the elderly or individuals who have a weakened immune system such as HIV patients are more likely to get infected.[1] Among the fungi which can cause fungal infections, *Candida albicans* is the species which leads to most of the fungal infections.[1, 3] *Candida albicans* is a yeast which commonly exist in humans. Their proliferation is restricted by the human immune system and other microorganisms. However, when the host immune defense system is compromised it becomes pathogenic.[4, 5]

Generally, antifungal drugs are targeted ergosterol.[6] As the major sterol of the fungal plasma membrane, ergosterol is significant for the cellular functions.[6, 7] The fluidity and integrity of the membrane requires ergosterol. In addition, ergosterol is also required for membrane bound enzymes to perform proper functions such as chitin synthetase, which plays a major role in cell wall biogenesis. [6]

There are two classes of drugs used to treat fungal infections, the polyenes and the azole-based drugs.[1, 6, 7] The polyenes such as amphotericin B and nystatin are target membranes containing ergosterol. Compared to cholesterol-containing

membranes, polyenes have a greater activity for ergosterol-containing membranes.[6] Studies show that these drugs can intercalate into membranes, forming channels that allow potassium ions to leak out of the cell, and destroying the proton gradient across the membrane. [6] The azole-based drugs including imidazoles and triazoles target lanosterol demethylase in the ergosterol biosynthesis pathway.[6, 7] However, there are problems with these antifungal drugs. Amphotericin B is the most effective antifungal agent, but it is toxic to human body. [1, 6] Some azole-based drugs can cause liver damage and endocrinological disorders.[1, 6, 8] In addition, the long term use of azole-based drugs to treat fungal infections has led to the emergence of many azole resistant strains.[8] Therefore, the investigation of new antifungal agents is significant.

## ***1.2 Antifungal peptide: histatin 5***

Several peptides and proteins with crucial antifungal properties have been studied such as cathelicidins, defensins and lactoferricins.[3, 6, 9] Among these are histatins: a family of low molecular weight, cationic, histidine-rich salivary peptides secreted by the parotid and submandibular glands.[10] The entire family of histatin peptides is encoded by only two human genes HTN1 and HTN2, and the products of these two genes are histatin 1 and histatin 3 (Table 1).[10, 11]

Histatin 1 has 38 amino acids with Ser-2 phosphorylated, while histatin 3 is composed of 32 amino acids. Histatin 1 and histatin 3 have similar amino acid

sequences.[10] The peptides in the histatin family are derived from histatin 1 and histatin 3 by specific proteolytic processes. [12]

**Table 1: Amino acid sequences of major histatins. (S<sup>P</sup> represents a phosphoserine residue)**

Histatin 1	DS <sup>P</sup> HEKRHHGYRRKFHEKHHSHREFPFYGDYDSNYLYDN
Histatin 3	DS HAKRHHGYKRKFHEKHHSHRGYRSNYLYDN
Histatin 5	DS HAKRHHGYKRKFHEKHHSHRGY

The histatin peptide with the most potential to be an antifungal agent is histatin 5, which consists of the first 24 amino acids of histatin 3 and has the highest killing activity against *Candida albicans* at physiological concentrations (15-30  $\mu$ M).[10, 13] Histatin 1, histatin 3, and histatin 5 constitute 85-90% of all histatin peptides in human saliva and histatin 5 is present at the highest concentration.[10, 11, 13, 14] Histatin 5 is also known to have antifungal activity against other fungi such as *Candida glabrata*, *Saccharomyces cerevisiae* and *Cryptococcus neoformans*.[14] Considering the significant antifungal activity of Histatin 5 and its non-toxic to humans, it is promising to investigate antifungal agents based on histatin 5.

### **1.3 Possible antimicrobial mechanism of histatin 5**

Even though the antifungal and antibacterial properties of the histatin peptides have been proven, the underlying mechanisms of action for histatin 5 are still unclear. The elucidation of the antifungal mechanism of histatin 5 could provide some new ideas

for the design of antifungal drugs. For most antifungal peptides, the generation of helical conformations in hydrophobic environments is crucial in their antifungal mechanisms.[9, 15, 16] Histatin 5 however is not act in this same way.[17] The proposed mechanism for the antifungal activity of histatin 5 is a multistep process, which includes damaging the cell membrane, disrupting the electron transport chain and generating reactive oxygen species (ROS).[11, 18-21]

Although the exact antifungal mechanism of histatin 5 is not identified, it has been proposed that in order to kill the yeast, histatin 5 must first interact with and pass through the cell wall.[11, 22-24] However, there is no conclusive information on how histatin 5 interacts with the cell wall, even though one study suggests that histatin 5 binds to the heat shock protein Ssa1/2 on the cell surface and then can be transported into the cell. [22, 23, 25] In addition, the exact intracellular target of histatin 5 is also unknown. One hypothesis is that histatin 5 targets the yeast mitochondria.[19, 20, 26] A previous study shows that non-respiring yeast cells are insensitive to histatin 5 which suggests that mitochondria may be crucial for the performance of the antifungal activity of histatins.[20] An early report suggests that after histatin 5 is transported to the cytosol it enters the mitochondria and interacts with coenzyme Q leading to the production of ROS in the electron transport chain which subsequently kills the yeast.[11, 20] In addition, the interaction between histatin 5 and the mitochondria can also induce the

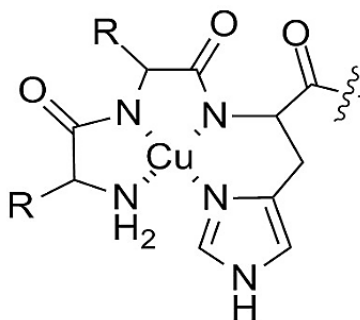
release of ATP into the cytoplasm which is proposed to be able to activate membrane ATP receptor and kill the yeast.[21] [2, 27]

Moreover, one mutant peptide of histatin 5, ATCUN-C16 (DSHAGYKRKRFHEKHSHRGY) has been synthesized and studied. The result shows that except for antifungal activity, this ATCUN-C16 peptide also has nuclease activity when copper, zinc ions and ascorbic acid are present.[28] This nuclease activity of ATCUN-C16 peptide may be due to the oxidative activities of the Cu-ATCUN complex.[28] This could be related to the proposed mechanism that histatin 5 disturbs the cell cycle by down-regulation of the expression of some proteins which participate in cell cycling.[2, 29] This oxidative nuclease activity in the presence of Cu(II) suggests a possible role of metal ions in the antifungal properties of histatin peptides.[28, 29]

#### **1.4 Metals and histatin 5**

Histatin 5 contains a zinc-binding motif His-Glu-X-X-His and an ATCUN (amino terminal Cu(II) and Ni(II)) motif.[30, 31] An ATCUN motif is a metal-binding amino acid sequence that exists in a variety of Cu(II)- and Ni(II)-binding proteins, such as albumins.[32, 33] The general structure of the ATCUN motif is H<sub>2</sub>N-X-X-His where X represents any amino acid except proline.[32, 33] The N-terminus amino group, the imidazole nitrogen of histidine and the two backbone nitrogens are involved in Cu(II) binding (Figure 1).[32, 33] In histatin 5, the ATCUN motif is the first three amino acids

(H<sub>2</sub>N-Asp-Ser-His) at the N-terminus. Previous studies show that histatin 5 has a high affinity for Cu(II) ( $K = 2.6 \times 10^7 \text{ M}^{-1}$ ) at the ATCUN site.[28, 31]



**Figure 1: General structure of ATCUN-Cu(II) complex.**

ATCUN motifs bind with Cu(II) using the lone pair of electrons on the imidazole nitrogen of histidine, the two backbone nitrogens and the N-terminus amino group.

How Cu(II) participates in the antifungal activity of histatin peptides is currently unknown. Copper is vital to many cellular metabolic processes including pigmentation, antioxidant activity and cell proliferation. Cellular uptake of copper in eukaryotes is achieved by the copper transport (CTR) proteins.[34] Previous data in the Franz lab show that the histidine residues in the human Ctr1 sequence play an important role in copper uptake.[34] The first 14 amino acids of human Ctr1 (MDHSHHMGMSYMS) have a high affinity for both Cu(II) and Cu(I).[34] The Cu(II) binding site of Ctr1 is an ATCUN site (H<sub>2</sub>N-Met-Asp-His); whereas the Cu(I) binding site is the two His-His residues with an additional histidine or methionine.[34] Considering that histatin 5 is also a histidine rich peptide that has an ATCUN motif (H<sub>2</sub>N-Asp-Ser-His) and an adjacent His-His site, the similarities between human Ctr1 peptide and histatin 5 are

interesting. In addition, despite the difference of the environment of these two peptides, it is attractive to study how the sequence of these two peptides affects the copper binding property and leading to their quite different functions.

### **1.5 Statement of objectives**

Previous studies in our lab suggest that human Ctr1 can bind with Cu(II) and Cu(I).[34] Early studies show that histatin 5 has a high affinity for Cu(II), but there are no clear studies related to its Cu(I) binding. Considering that metal-ion binding may be related to the antifungal properties of histatin 5, analysis of the Cu(II) and Cu(I) binding properties of histatin 5 may give us a new understanding about the mechanism of its antifungal activity.

The goal for this project is to study the copper binding properties of histatin 5 and analyze the role this copper binding property plays in the antifungal activity of histatin 5. Here, a series of histatin 5 derivatives containing the first 11 amino acids have been constructed and studied. Cu(II) and Cu(I) binding studies were performed by UV-Vis spectroscopy and mass spectrometry. In addition, ROS formation was been investigated.

## 2. Spectroscopic studies of copper binding of histatin 5 model peptides

### 2.1 Background and significance

Due to the diminishing effectiveness of antifungal drugs, the rate of fungal infections is increasing. *Candida albicans* is a genus of yeast that causes most human fungal infections.[1, 6] The increasing resistance of fungal strains to antifungal drugs raises the need for new antifungal agents. Based on these reasons, histatin 5, which exists in human saliva and has significant killing activity to *Candida albicans*, has been noticed.

It is known that the metal binding properties of histatin 5 may relate to its antifungal activity.[29-31] Histatin peptides have the potential to complex with metal ions.[35] Histatin 5 contains a zinc-binding motif and an ATCUN motif resulting in a high affinity for Zn(II) and Cu(II).[30, 31] In addition, histatin 5 also can bind with Ni(II), Ca(II) and Fe(II).[30, 31] The order of metal binding affinity to histatin 5 is Cu(II)>Ni(II)>Zn(II) in aqueous solution whereas Ca(II) and Fe(II) can only weakly bound to histatin 5.[31]

It is known that histatin 5 can bind with Cu(II) and has an absorbance band at 525 nm which is the typical Cu(II)-ATCUN peak.[32, 33] It is also reported that a short ATCUN-containing synthetic model peptide of histatin 5 has pro-oxidant activity.[29] It was speculated that this formation of ROS by Cu(II) and histatin 5 may contribute to its antifungal mechanism.[29]

While Cu(II) binding has been documented for histatin 5, there are no reported studies related to its Cu(I) binding. The goal of this chapter is to study the Cu-binding properties of synthetic peptides corresponding to the histatin peptides sequence. Here, a series of histatin 5 derivatives composed of the first 1-11 residues of the native peptide have been investigated, specifically with regards to the importance of each histidine for Cu(II) and/or Cu(I) binding. UV-Vis spectroscopy is used to study Cu(II) and Cu(I) binding.

## **2.2 Results**

### **2.2.1 Model peptide design**

Model peptides of the first 11 amino acids of histatin 5 were prepared on a peptide synthesizer by using Fmoc-based solid-phase peptide synthesis methodology and purified by HPLC. ATCUN-HH contains the first 11 residues of histatin 5 plus a tryptophan at the C-terminus which provides a chromophore for accurate determination of peptide concentration by absorption spectroscopy. To determine the amino acids that are important for copper binding, histidine residues in the ATCUN-HH peptide were systematically substituted for alanine. The peptide in which two histidines in positions 7 and 8 are replaced by alanine is called H7A/H8A, whereas peptides with a single histidine replaced at position 3, 7 or 8 are called H3A, H7A and H8A, respectively. Histatin 8 is the last 12 residues of histatin 5, however, the distance between the ATCUN motif and the His-His site in histatin 8 is shorter than the ATCUN-HH peptide. Histatin

8 was synthesized to identify whether this will affect the copper binding property of the peptide. F9AA contains the first nine amino acids of histatin 5, and notably contains no tyrosine or tryptophan residues.

**Table 2: Names and sequences of synthesized peptides\***

Peptide name	Amino acid sequence
ATCUN-HH	D-S-H-A-K-R-H-H-G-Y-K-W
H7A	D-S-H-A-K-R-A-H-G-Y-K-W
H3A	D-S-A-A-K-R-H-H-G-Y-K-W
H7A/H8A	D-S-H-A-K-R-A-A-G-Y-K-W
H8A	D-S-H-A-K-R-H-A-G-Y-K-W
F9AA	D-S-H-A-K-R-H-H-G
Ctrl	M-D-H-S-H-H-M-G-M-S-Y-M-D-S
Histatin 8	K-F-H-E-K-H-H-S-H-R-G-Y

\* All peptides prepared as the free amine at the N-terminus (H<sub>2</sub>N) and C-terminal amide.

### 2.2.2 Cu(II) binding study

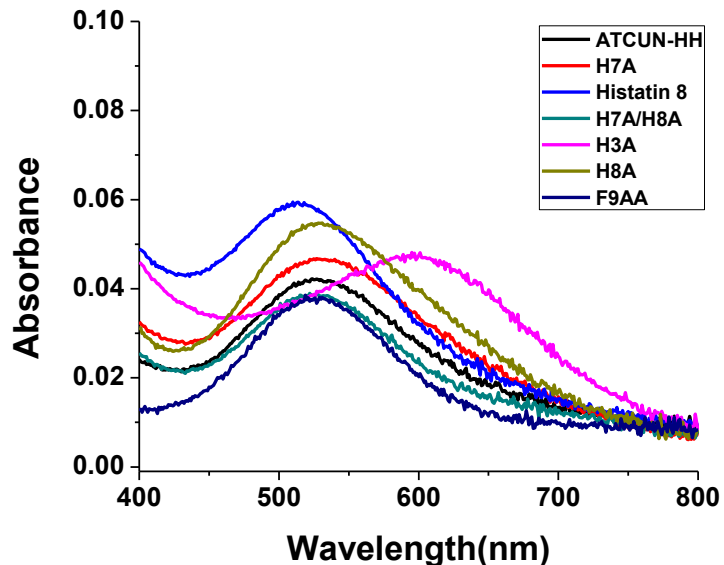
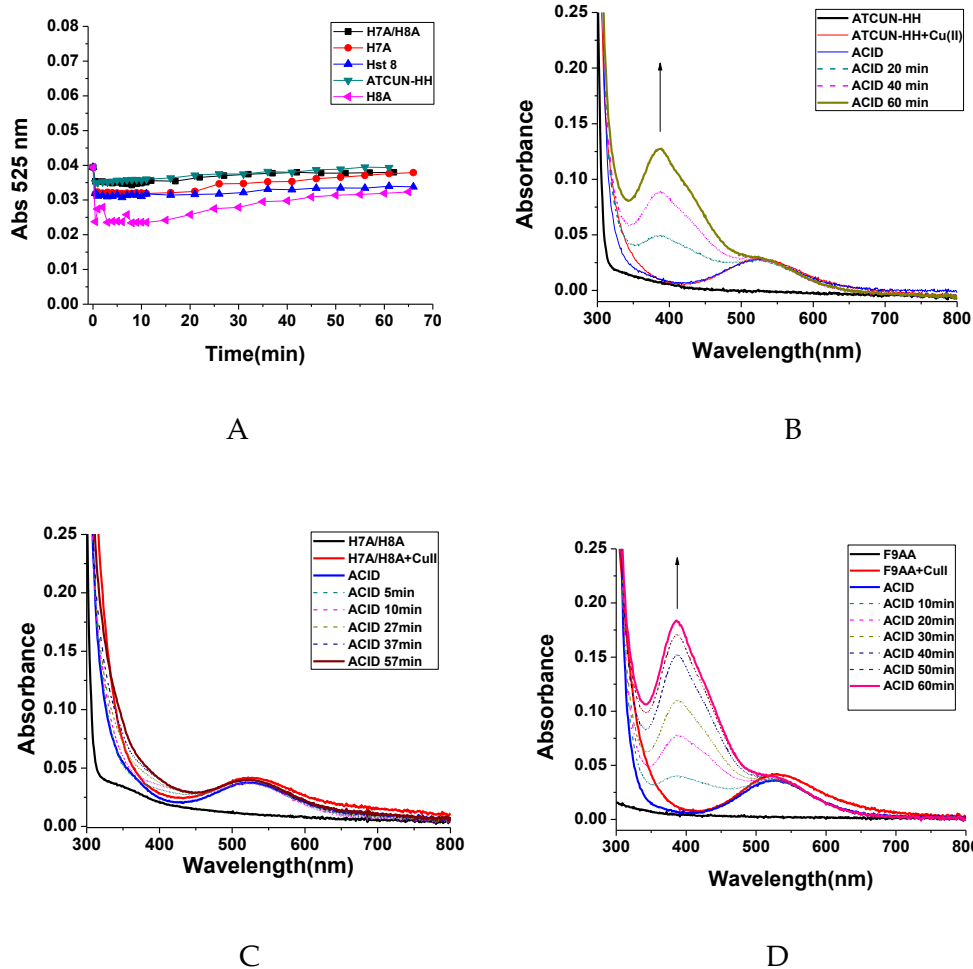


Figure 2: UV-Vis spectra of peptide-Cu(II) complexes.

Absorbance spectra of 0.3 mM model peptides (ATCUN-HH, H7A, H7A/H8A, H3A, H8A, F9AA and histatin 8) with 1 equivalent of  $\text{CuSO}_4$  at pH 7.4 in 50 mM HEPES buffer. The peptides that have an ATCUN motif show a Cu(II)-ATCUN absorbance band near 525 nm.

A previous report shows that histatin 5 has a high affinity for Cu(II) ( $K = 2.6 \times 10^7 \text{ M}^{-1}$ ). [30, 31] In order to confirm that the first 11 amino acids of histatin 5 also have this property, Cu(II) binding of the model peptides was investigated by UV-Vis spectroscopy. Figure 2 shows the spectrum for the model peptides with Cu(II) in 50 mM HEPES buffer at pH 7.4. The ATCUN-HH peptide with  $\text{CuSO}_4$  has an absorbance band near 525 nm which is characteristic of an albumin-like distorted square planar coordination environment with Cu(II). [32] Substitution of the histidine at the seventh or

eighth position causes no significant change in the peptide-Cu(II) spectrum (Figure 2). This indicates that neither the histidine residue in position 7 nor 8 are participate in the Cu(II) binding. In contrast, replacement of histidine in the third position such as in the H3A peptide results in an absorbance shift, suggesting that the coordination environment is different in this mutant peptide. The Cu(II)-histatin 8 and Cu(II)-F9AA spectra also show an absorbance near 525 nm because these two peptides also have an ATCUN motif. This data indicates that the model peptides containing an ATCUN motif can bind Cu(II). This finding is consistent with previous studies that the ATCUN motif of histatin 5 has a high affinity for Cu(II).



**Figure 3: UV-Vis spectra of peptides with 1 equiv  $\text{CuSO}_4$  (0.3 mM) and 1 mM ascorbic acid in 50 mM HEPES buffer.**

A) Comparison of the changes in absorbance at 525 nm. B) ATCUN-HH + Cu(II) + ascorbic acid. C) H7A/H8A + Cu(II) + ascorbic acid. D) F9AA + Cu(II) + ascorbic acid. Spectrum A shows that the expected ATCUN-HH-Cu(II) absorbance band at 525 nm have nearly no change for the given model peptides when reducing agent present. There is a new peak generated near 390 nm after adding ascorbic acid to Cu(II)-ATCUN-HH and Cu(II)-F9AA solution.

In order to verify whether a reducing agent will reduce Cu(II) to Cu(I) in the presence of model peptides, peptide-Cu(II) (1:1, 0.3 mM) solutions with excess ascorbic

acid (1 mM) in 50 mM pH 7.4 HEPES were monitored by UV-Vis spectroscopy. This experiment was conducted with ATCUN-HH, histatin 8, H7A, H7A/H8A, H8A, F9AA and Ctr1 peptide.

Figure 3A shows that there is no significant change in the Cu(II)-ATCUN binding site for any of the histatin model peptides tested. This indicates that even with excess reducing agent present, the ATCUN motif of the peptides can still bind with Cu(II). However, the addition of ascorbic acid generates an obvious peak around 390 nm for the ATCUN-HH and F9AA peptide which is not expected for this experiment (Figure 3B, 3D). This peak was not observed for H7A, H7A/H8A and H8A peptides. Also, the addition of excess ascorbic acid to the Ctr1-Cu(II) solution also did not produce an absorbance band around 390 nm (Appendix A.1). Further studies need to be employed to verify which species leads to the observed absorbance band around 390 nm.

### 2.2.3 Cu(I) binding study

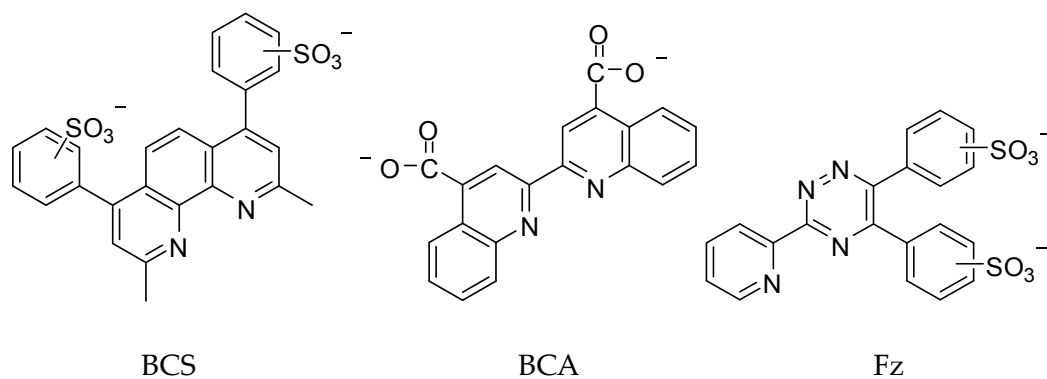
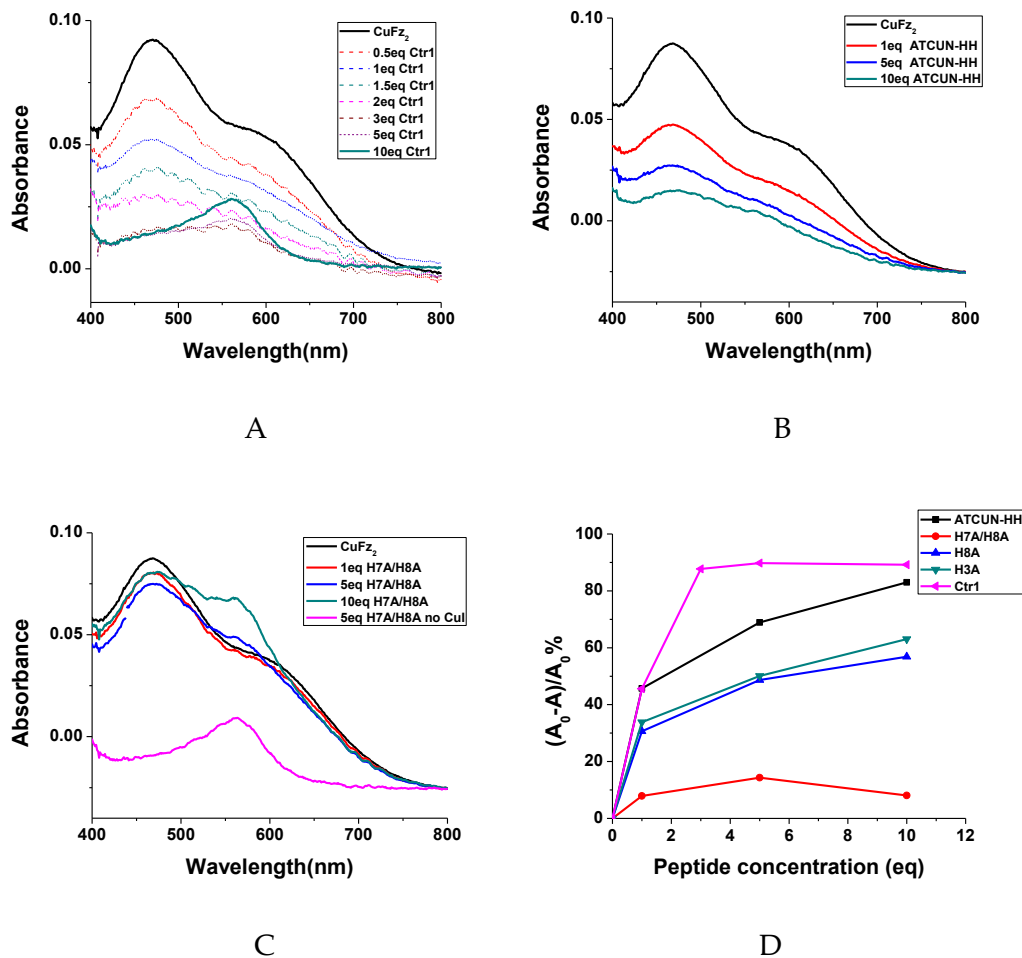


Figure 4: Structure of BCS, BCA, ferrozine (Fz)

Directly determining Cu(I) binding affinity is often difficult. Therefore, Cu(I) colorimetric indicators such as BCS (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonic acid,  $\log\beta_2=19.8$ ), BCA (bicinchoninic acid,  $\log\beta_2=14.7$ ), and Fz (ferrozine,  $\log\beta_2=11.6$ ) are frequently used to obtain Cu(I) binding affinity by competition.[36-38] In this study, a first attempt was completed using BCA as a competitive ligand to study the Cu(I) binding of model peptides. When adding Ctr1 to  $[\text{Cu(I)(BCA)}_2]^{3-}$  solution in a nitrogen-filled glove box, the absorbance of  $[\text{Cu(I)(BCA)}_2]^{3-}$  at 562 nm decreased (Appendix A.5). This result is consistent with our lab's previous studies showing that this Ctr1 peptide can compete with BCA for Cu(I) binding.[34] However, for other model peptide of histatin 5, the affinity of BCA for Cu(I) is too high to allow effective competition.

The weaker ligand ferrozine (Fz) was then used as a competitive ligand to determine the Cu(I) binding property of model peptides. Fz is a well-known colorimetric indicator for Fe(II) which has also found utility as a Cu(I) colorimetric indicator.[36] The  $[\text{Cu(I)(Fz)}_2]^{3-}$  complex in solution has an absorbance band at 470 nm ( $\epsilon=4320\text{ M}^{-1}\text{cm}^{-1}$ ).[36] In this study, solutions of  $[\text{Cu(I)(Fz)}_2]^{3-}$  were titrated with up to 10 equivalents of model peptides.



**Figure 5: Different peptides compete with Fz for Cu(I) in glove box.**

In this experiment,  $[\text{Cu(I)(CH}_3\text{CN)}_4]\text{PF}_6 = 20 \mu\text{M}$ ,  $\text{Fz} = 50 \mu\text{M}$ , 50 mM HEPES buffer pH=7.4. A) Ctr1 compete with Fz for Cu(I), tried 0.5, 1, 1.5, 2, 3, 5, 10 equivalents of Ctr1 peptide. B) ATCUN-HH compete with Fz for Cu(I), tried 1,5,10 equivalents of ATCUN-HH. C) H7A/H8A compete with Fz for Cu(I), tried 1,5,10 equivalents of H7A/H8A. D) Comparison the Cu(I) binding ability of model peptides.

Previous data in our lab shows that the first 14 amino acids of the human Ctr1 have a high affinity for Cu(I).[34] In this study we first tried this Ctr1 peptide to titrate a  $[\text{Cu(I)(Fz)}_2]^{3-}$  solution in nitrogen-filled glove box. As shown in Figure 5A, competition

can be observed by the decrease of absorbance at 470 nm with the addition of Ctr1 peptide. There is a weak increase at 562 nm which indicates the formation of  $[\text{Fe(II)(Fz)}_3]^{4-}$  complex due to a small amount of residual Fe in the sample.[36] In addition, after adding 3 equivalents of Ctr1, the absorbance band at 470 nm disappeared which indicates that Ctr1 has a strong affinity for Cu(I), which corresponded to our previous study.

In order to evaluate if the histidines in histatin 5 enable Cu(I) binding, ATCUN-HH, H3A, H7A/H8A, H8A were tested in this competition assay. Figure 5D shows that ATCUN-HH, H3A and H8A can compete with Fz for Cu(I). However, after the addition of 10 equivalents of ATCUN-HH, H3A or H8A, the absorbance of  $[\text{Cu(I)(Fz)}_2]^{3-}$  at 470 nm has still not disappeared completely, as it had for Ctr1. This result indicate that Ctr1 has stronger Cu(I)-binding affinity than ATCUN-HH , H3A and H8A. Furthermore, the data plotted in Figure 5D reveals that the decrease in absorbance at 470 nm is more significant for the ATCUN-HH peptide than for either the H3A or H8A peptides which indicates that the Cu(I)-affinity of ATCUN-HH is greater than that of H3A or H8A. This finding suggests that all 3 histidines of the ATCUN-HH peptide contribute to the appreciable binding.

Figure 5C shows that the addition of 10 equivalents of H7A/H8A cannot compete with Fz for Cu(I); the absorbance at 470 nm does not significantly change and the absorbance at 562 nm increases which is due to residual Fe present in the peptide

sample. Therefore, the substitution of any one of the three histidines in position 3, 7 or 8 can reduce the Cu(I) binding affinity of ATCUN-HH. The remove of the two histidine residues in position 7 and 8 can lead to the complete loss affinity for Cu(I). Histidines are important for the Cu(I) binding of histatin 5 model peptides at pH 7.4.

### **2.3 Summary**

In this study, UV-Vis spectra show that synthesized histatin 5 model peptides which contain an ATCUN motif can bind with Cu(II). This finding confirms the hypothesis that the ATCUN-HH peptide would bind Cu(II) similar to histatin 5. Moreover, with excess ascorbic acid present in the solution, the ATCUN-Cu(II) absorbance band at 525 nm has no significant changes which indicates that the ATCUN peptides can still bind with Cu(II) even in the presence of a strong reducing agent.

Previous studies in our lab show that the histidine residues in Ctr1 play an important role in the interaction with Cu(I).[34] For histatin 5 model peptides, the result is similar. In this chapter it was shown that the histidine residues of that ATCUN-HH peptide has appreciable affinity for Cu(I). It is clear from the experiments discussed above that ATCUN-HH can bind both oxidation states of copper with significant affinity.

In summary, this chapter provides direct evidence that model peptides of histatin 5 can form complexes with Cu(II) and Cu(I). These properties may be related to the established antifungal activity of histatin 5. It is interesting that after adding excess ascorbic acid to an ATCUN-HH-Cu(II) solution, there is a new peak generated near 390

nm. The next chapter will describe a comprehensive study of the conditions that favor the formation of this new species.

## **2.4 Materials and methods**

### **Reagent and method:**

Most of the reagents and solvents were purchased from Sigma Aldrich. Fmoc-protected amino acids were purchased from Novabiochem and Chem-Impex International.

Standard Fmoc-based solid-phase peptide synthesis strategy was used to synthesize model peptides on a Protein Technologies PS3 peptide synthesizer. A Waters Delta 600 HPLC equipped with a Water semi-preparative C-8 column was used to purify peptides. Lyophilization of all the model peptides was completed by using a Labconco Lyophilizer. Varian Cary 50 Conc UV-Visible spectrophotometer was used to collect all the UV-Vis absorbance data. LC/MS spectra were collected on an Agilent 1100 Series spectrometer utilizing an electrospray ionization source coupled with an ion trap detector.

### **Preparation of stock solution:**

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was dissolved in Nanopure water (18.0 M $\Omega$ ) to get the Cu(II) stock solution, the concentration of Cu(II) stock solution was determined by EDTA (Ethylenediaminetetraacetic acid) titration to a murexide (Fischer) endpoint.

The peptide stock solutions were prepared by dissolving lyophilized peptide in Nanopure water (18.0 MΩ). The Edelhoch method was used to decide the concentrations of peptide solution.[39] In general, peptide stock solution was added into 1 ml of 8 M Urea solution, the absorbance at 280 nm was monitored by UV-Vis spectroscopy. The extinction coefficients for tyrosine ( $\epsilon=1490 \text{ M}^{-1}$ ) and tryptophan ( $\epsilon=5500 \text{ M}^{-1}$ ) at 280 nm were used to calculate the concentration of peptide stock solution.[39]

### **Synthesis**

Fmoc-PAL-PEG-PS resin (Applied Biosystems) was used to synthesis all the model peptides. Amino acid coupling was achieved by using HBTU (O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate; Novabiochem) and the coupling reagents (N-methylmorpholine (NMM) in N,N'-dimethylformamide ). Fmoc deprotection was achieved by using deprotection reagents which is 20% piperidine in DMF (v/v). After the peptide was synthesized by peptide synthesizer, the resin was washed by acetic acid, dichloromethane and methanol three times. 10 ml trifluoroacetic acid (TFA), 150  $\mu\text{l}$  ethane dithiol (EDT), and 150  $\mu\text{l}$  triisopropylsilane (TIS) were added to the resin and mixed for 4 hour under  $\text{N}_2$ . 125-150  $\mu\text{l}$  of EDT and 125-150  $\mu\text{l}$  of bromotrimethylsilane (TMSBr) were added during the final 45 minutes. After this,  $\text{N}_2$  was used to evaporate the TFA solution. When the volume of solution was less than 5 mL, the peptides were washed by diethyl ether and centrifuged 3 times. Peptides were purified by HPLC on a C-8 column with a linear 40 minutes gradient from 0-60 %

acetonitrile in water with 0.1 % TFA. The purity of model peptides was verified by LC-MS.

### **Cu(I) binding study**

Cu(I) stock solutions were prepared by dissolving  $[\text{Cu}(\text{CH}_3\text{CN})_4]\text{PF}_6$  in anhydrous acetonitrile in nitrogen-filled glove box. The concentration of Cu(I) stock solutions were determined spectrophotometrically by titration with excess BCA solution in the glove box.  $\text{Cu}(\text{I})(\text{BCA})_2$  has absorbance at 562 nm ( $\epsilon=7900 \text{ M}^{-1}$ ), and the absorbance data at 562 nm was used to calculate Cu(I) concentration.[34]

Cu(I) competition assay was performed at pH 7.4 in 50 mM HEPES buffer, the concentration of Cu(I) was 20  $\mu\text{M}$  and the concentration of ferrozine (Fz) was 50  $\mu\text{M}$ . In this assay, the use of 1, 5, 10 equivalents of model peptides to compete with Fz is monitored by UV-Vis spectroscopy. If the peptide competes with Fz, the absorbance at 470 nm will decrease. All the Cu(I) binding study were conducted in a nitrogen-filled glove box, and the solution were all deoxygenated.

### **3. Investigate the solution conditions that favor the 390 nm spectral feature**

#### ***3.1 Background and significance***

Early studies of the metal-binding properties of histatin 5 revealed that it can bind with Zn(II), Cu(II) and Ni(II).[31] In the previous chapter, UV-Vis spectroscopy studies showed that model peptides that have an ATCUN motif can bind with Cu(II). This finding confirms the hypothesis that a shorter ATCUN-HH peptide binds Cu(II) in a similar fashion as the longer native histatin 5. In addition the Fz competition assay showed that ATCUN-HH can compete with Fz for Cu(I) and establishes that histatin 5 peptides can bind with Cu(I). Therefore, it is clear that ATCUN-HH can bind Cu(II) and Cu(I), furthermore, all 3 histidine residues contribute to optimal competition with Fz for Cu(I).

Because these model peptides can bind either oxidation state of copper, it would be interesting to study the reduction of Cu(II)-peptide complexes. It is important to note that when excess ascorbic acid is present, a new peak near 390 nm appears in the ATCUN-HH peptide/Cu(II) solution. This new peak is only formed for the ATCUN-HH and F9AA, no other histatin model peptides have this property. This result suggests that the sequence of the histatin 5 model peptide affects the redox properties of the peptide-Cu(II) complexes.

The cell space is a reducing environment replete with reducing agents including ascorbate, cysteine, and NADH. Copper ions could be acquired from a copper pool in

yeast mitochondria.[20, 29, 40] Considering in the presence of excess ascorbic acid, only the ATCUN-HH and F9AA peptides have this new absorbance at 390 nm. This new species may have some relationship with the antifungal activity of histatin 5.

The aims for this chapter are to study the solution conditions and peptide sequences that favor observation of this 390 nm spectral feature formed in the reaction of ATCUN-HH with Cu(II) when excess ascorbic acid is present. UV-Vis spectroscopy and mass spectrometry methods were used in this chapter to interrogate the influence of factors such as oxygen, the effect of different reducing agent, the stoichiometry of peptide and Cu(II) on the formation of species formed at 390 nm.

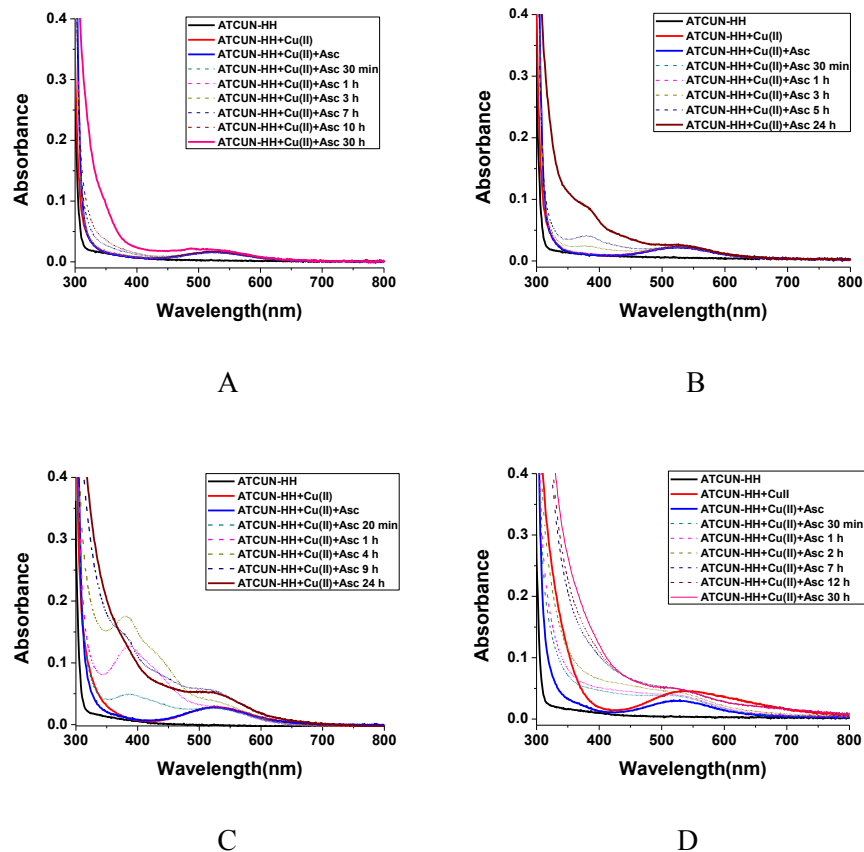
## **3.2 Results**

### **3.2.1 The effect of copper concentration**

The studies in chapter 2 show that a new peak near 390 nm formed in the solution containing excess ascorbic acid, ATCUN-HH and Cu(II). In order to elucidate which species lead to the formation of this peak we first want to know whether the ratio of peptide to Cu(II) will affect the absorbance at 390 nm. In this experiment, ATCUN-HH peptide concentration was kept constant at 0.3 mM with 1 mM ascorbic acid, while the copper concentration varied from 0.5-2 equivalents of the peptide. Samples were prepared in 50 mM HEPES buffer pH=7.4.

Figure 6 shows the UV-Vis spectra result when 1 mM ascorbic acid is combined with the peptides and 0.5, 0.6, 1, 2 equivalents of CuSO<sub>4</sub>. The sample of ATCUN-HH

peptide containing only 0.5 equivalents of Cu(II) do not develop an absorption peak at 390 nm, although there is a slight increase in absorbance generally below 400 nm. In contrast, when the copper concentration is 0.8-1.5 equivalents of peptide, a distinct peak forms within minutes at  $\lambda_{\max}$  centered at 390 nm. This new feature grows in over the course of 1-2 hours and stabilizes at a similar intensity for these samples containing 0.8-1.5 Cu(II) (Appendix A.3). The absorbance at 390 nm is much weaker when 0.6 equivalents copper is present in the solution (Figure 6B). When the Cu(II) : ATCUN-HH ratio increases to 2:1, a distinct peak is not observed, although the intensity between 350-500 nm increases generally (Figure 6D).

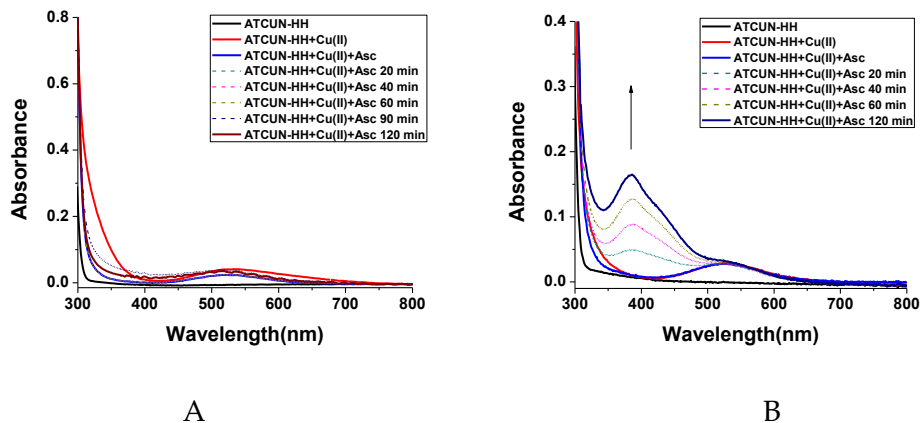


**Figure 6: UV-Vis spectra of ATCUN-HH peptide with different concentration of Cu(II) in the presence of excess of ascorbic acid.**

ATCUN-HH = 0.3 mM, ascorbic acid = 1 mM, 50 mM HEPES buffer pH 7.4. A) 0.5 equivalents of Cu(II), B) 0.6 equivalents of Cu(II) C) 1 equivalents of Cu (II) D) 2 equivalents of Cu(II). The formation of the absorbance at 390 nm can be effected by the ratio of ATCUN-HH to Cu(II).

These experiments show that the ratio of Cu(II) to ATCUN-HH peptide has some effect on the formation of the new peak near 390 nm, and that the formation of this feature is optimal when ratio of Cu : peptide close to 1.

### 3.2.2 Generation of new species requires oxidizing agents

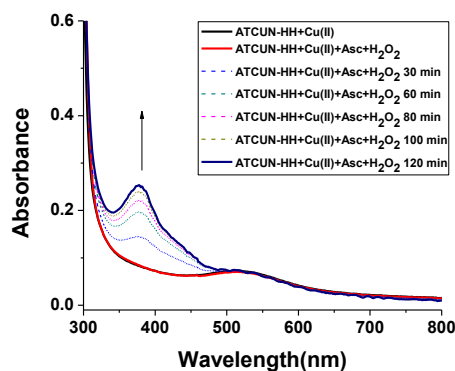


**Figure 7: UV-Vis spectra of ATCUN-HH peptide, Cu(II) and excess ascorbic acid in anaerobic conditions and aerobic conditions.**

ATCUN-HH peptide and Cu(II) concentration is 0.3 mM, ascorbic concentration is 1 mM, experiments performed in 50 mM HEPES buffer, pH=7.4. A) Experiment conducted in anaerobic conditions. B) Experiment conducted on the benchtop in aerobic conditions. The generation of the new peak at 390 nm requires the presence of oxygen.

In order to test whether the formation of this new species requires oxygen, samples of peptide with equimolar  $\text{CuSO}_4$  and excess ascorbic acid were prepared in de-oxygenated solvents and monitored spectrophotometrically in a nitrogen-filled anaerobic glove box. Figure 7 shows the comparison of this experiment conducted under anaerobic and aerobic conditions. In the absence of  $\text{O}_2$ , the addition of 1 mM ascorbic acid does little to change the spectrum of the Cu(II)-peptide species (Figure 7A). Notably, no new feature appears at 390 nm, and the broad absorbance centered at 525 nm characteristic of Cu(II)-ATCUN species remains intact. However, in the presence of air, the peak at 390 nm readily forms upon mixing with 1 mM ascorbic acid (Figure 7B).

These experiments indicate that the generation of the new species at 390 nm requires oxygen.



**Figure 8: UV-Vis spectrum of ATCUN-HH, Cu(II) with excess ascorbic acid and H<sub>2</sub>O<sub>2</sub> in anaerobic conditions.**

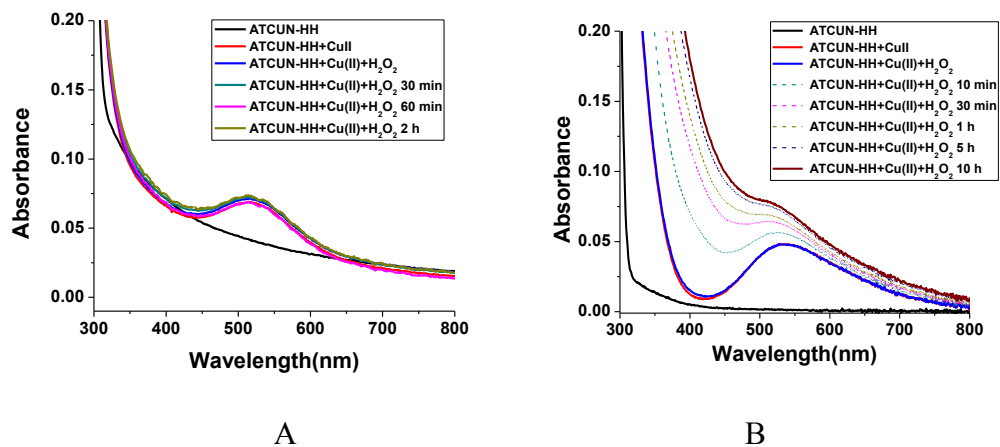
ATCUN-HH = 0.3 mM, Cu(II) = 0.3 mM, ascorbic acid = 1 mM, hydrogen peroxide concentration is 1 mM. Experiment was conducted in 50 mM HEPES buffer pH=7.4 in anaerobic conditions.

In order to test whether other oxygen-containing species could facilitate formation of the 390 nm feature, 1 mM hydrogen peroxide and 1 mM ascorbic acid were added to ATCUN-HH/Cu(II) solution in 50 mM HEPES buffer in the nitrogen-filled glove box and monitored by UV-Vis spectroscopy. Figure 8 shows that after adding excess H<sub>2</sub>O<sub>2</sub> and ascorbic acid a peak forms at 390 nm with an absorbance that grows in stronger than in samples that are prepared under ambient aerobic conditions (compare to Figure 6C for example). This experiment indicates that H<sub>2</sub>O<sub>2</sub> can facilitate formation of the new species even in the absence of O<sub>2</sub>. Furthermore, the Cu-catalyzed aerobic

oxidation of ascorbic acid is known to generate  $\text{H}_2\text{O}_2$  in situ, which suggest that  $\text{H}_2\text{O}_2$  may in fact be the active agent in the aerobic experiments as well.

### **3.2.3 The effect of ascorbic acid**

The previous experiment clearly showed that  $\text{H}_2\text{O}_2$  is capable of generating the 390 nm feature from solutions of Cu(II) and ATCUN-HH peptide, at least in the presence of ascorbic acid. Several experiments were therefore conducted to test the dependence of these reactions on ascorbic acid. As shown in Figure 9A, the addition of 1 mM  $\text{H}_2\text{O}_2$  to 0.3 mM Cu(II)-ATCUN-HH under anaerobic conditions does not noticeably change the UV-Vis spectrum characteristic of the Cu(II)-ATCUN-HH species. When the same experiment is conducted under aerobic conditions (Figure 9B) a general and significant increase of absorbance grows in between 400-500 nm, indicative of some kind of non-specific reactivity. Although, a distinct feature at 390 nm is not apparent. The anaerobic experiment here is the most revealing, and suggests that  $\text{H}_2\text{O}_2$  does not oxidizing or reduce the Cu(II) center complexed by ATCUN-HH and is not able to generate the 390 nm feature directly from reaction with the Cu(II) peptide.



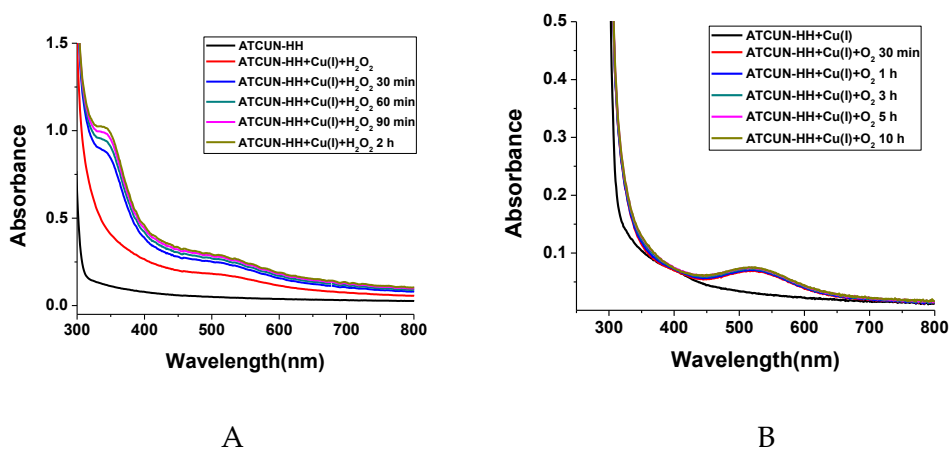
**Figure 9: UV-Vis spectra of ATCUN-HH with Cu(II) in the presence of excess H<sub>2</sub>O<sub>2</sub> in anaerobic and aerobic conditions.**

ATCUN-HH = 0.3 mM, Cu(II) = 0.3 mM, H<sub>2</sub>O<sub>2</sub> = 1 Mm, 50 mM HEPES buffer pH = 7.4. A) Experiment conducted in anaerobic conditions. B) Experiment conducted in aerobic conditions.

The failure of H<sub>2</sub>O<sub>2</sub> to generate the new peak at 390 nm without ascorbic acid present may be because the formation of the new peak requires a reducing agent to form Cu(I). In order to test this idea two experiments were conducted. The first was to add excess hydrogen peroxide to a Cu(I)-ATCUN-HH solution in anaerobic conditions, and the second was to make a Cu(I)-ATCUN-HH solution in the glove box then take the solution out and bubble oxygen into the solution.

Figure 10A shows that the anaerobic addition of hydrogen peroxide or oxygen to solutions containing Cu(I)-ATCUN-HH solution generate two new features. A broad absorbance appears around 525 nm, which may be due to formation of Cu(II)-ATCUN, while a distinct peak at 350 nm concomitantly appears. However, this 350 nm feature

appears not the same as the 390 nm feature, its appearance indicates that Cu(I)-ATCUN-HH does react directly with H<sub>2</sub>O<sub>2</sub> to generate a new species. In contrast, exposure of Cu(I)-ATCUN-HH directly to O<sub>2</sub> results only in the formation of the 525 nm feature indicative of Cu(II)-ATCUN, with no indication of an intermediate, at least under these timescales. While these combined results indicate that the generation of the 390 nm new species requires ascorbic acid, the direct reaction of Cu(I)-ATCUN-HH by H<sub>2</sub>O<sub>2</sub> to generate a new species at 350 nm is interesting.

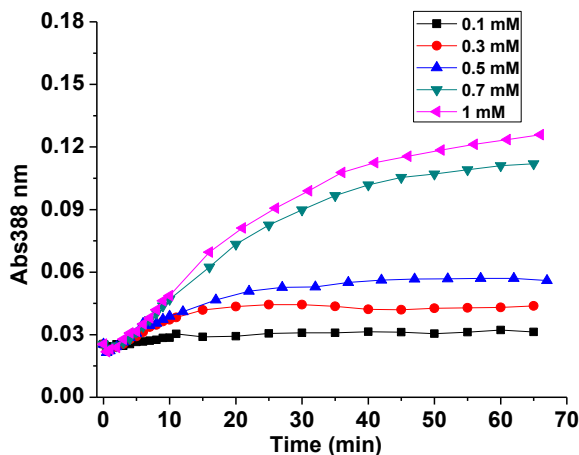


**Figure 10: UV-Vis spectra of ATCUN-HH and Cu(I) upon addition of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>.**

ATCUN-HH= 0.3 mM, Cu(I)= 0.3 mM, experiments conducted in 50 mM HEPES buffer, pH= 7.4. A) ATCUN-HH and Cu(I) in the presence of H<sub>2</sub>O<sub>2</sub> in anaerobic conditions. B) ATCUN-HH-Cu(I) solution was made in glove box, after took it out from glove box bubbling oxygen to the solution. These experiments show that without ascorbic acid present in the solution, the peak at 390 nm cannot be formed.

To further probe the ascorbic acid dependence on formation of the 390 nm peak, different concentrations of ascorbic acid were added to a Cu(II)-peptide. In this experiment, aliquots of 0.3 mM, 0.5 mM, 0.7 mM and 1 mM ascorbic acid were added to

Cu(II)-ATCUN-HH solutions in aerobic conditions. Figure 11 shows that the absorbance band around 390 nm increased when the concentration of ascorbic acid increased.



**Figure 11: Changes of UV-Vis absorption at 388 nm overtime of ATCUN-HH, Cu(II) in the presence of different concentrations of ascorbic acid.**

ATCUN-HH= 0.3 mM, Cu(II) = 0.3 mM, 50 mM HEPES buffer pH 7.4. The concentration of ascorbic acid is 0.1 mM, 0.3 mM, 0.5 mM, 0.7 mM, 1 mM. The absorption near 390 nm increased as a function of ascorbic acid concentration.

### 3.2.4 The effect of different reducing agents

The previous studies show that ascorbic acid is required to generate the new species, the absorbance of which increases at 390 nm as the concentration of ascorbic acid increases. It is interesting to study whether this new species is specific to ascorbic acid or if it is also formed when adding other reducing agents.

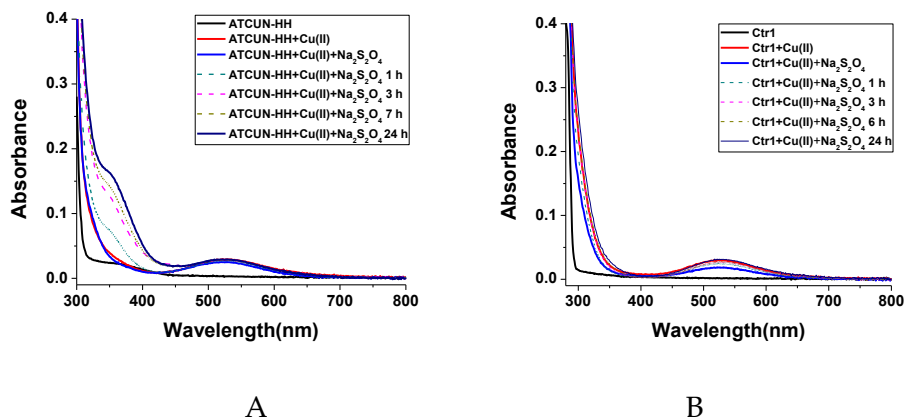
Sodium dithionite was used as an alternative reducing agent to ascorbic acid in these experiments. Peptide-Cu(II) (1:1, 0.3 mM) solutions with excess sodium dithionite

(1 mM) in 50 mM pH 7.4 HEPES were monitored by UV-Vis spectroscopy. Figure 12 shows the comparison of ATCUN-HH and Ctr1 peptide.

Figure 12A shows that the 525 nm absorption band of Cu(II)-ATCUN-HH does not change upon addition of excess sodium dithionite, while a new feature with weak absorbance around 370 nm grows in within the first hour. This new feature at 370 nm has a different appearance and  $\lambda_{\text{max}}$  compared to the 390 nm feature found in the ascorbic acid reactions. The mutant peptides H3A and H7A/H8A under the same conditions also show growth of absorbance below 400 nm in the presence of sodium dithionite, but the feature takes slower to develop and has weaker intensity compared to ATCUN-HH (Appendix A.4).

In contrast to the observations with ATCUN-HH, the 525 nm signal of Cu(II)-Ctr1 initially loses intensity upon addition of sodium dithionite before recovering its original intensity over time (Figure 12B), suggesting that sodium dithionite is able to reduce Cu(II) to Cu(I), but over time in air reoxidation to Cu(II) reforms Cu(II)-Ctr1. Also unlike the ATCUN-HH example is that no few feature below 400 nm appears in spectra of Cu(II), Ctr1 and sodium dithionite in the presence of air. Inexplicably, the Cu(II)-F9AA peptide sample does not show any signs of reaction with sodium dithionite (Appendix A.4). The F9AA peptide does not contain any tyrosine or tryptophan residues, but its reactivity with Cu(II) and ascorbic acid is nearly identical to that of ATCUN-HH with respect to formation of the 390 nm feature. Its different reactivity with sodium

dithionite is therefore surprising, and further suggests that the 370 nm feature observed in the ATCUN-HH/dithionite reaction is unrelated to the 390 nm feature observed in reactions with ascorbic acid.



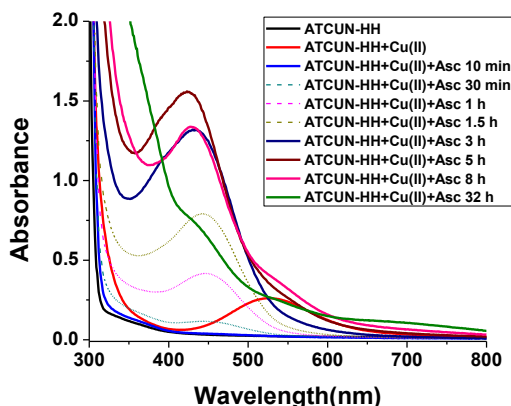
**Figure 12: UV-Vis spectra of peptide, Cu(II) in the presence of excess sodium dithionite.**

ATCUN-HH = 0.3 mM, Ctr1 = 0.3 mM, Cu(II) = 0.3 mM, sodium dithionite = 1 mM, experiments conducted in 50 mM HEPES buffer pH=7.4. A) ATCUN-HH + Cu(II) + Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, B) Ctr1 + Cu(II) + Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>.

### 3.2.5 The effect of component concentrations

In an attempt to generate a high-concentration sample of the species absorbing at 390 nm for further spectroscopic analysis, a sample was prepared in air containing 2.4 mM Cu(II)-ATCUN-HH and 8 mM ascorbic acid in 50 mM HEPES buffer, pH=7.4. Figure 13 shows the unexpected result. Instead of generating the 390 nm feature at higher intensity, a strong absorbance near 420 nm formed after the addition of ascorbic acid and this peak shifted to the left over 32 hours. Also, the absorbance band of the

ATCUN-Cu(II) binding site around 525 nm disappeared initially upon addition of ascorbic acid which initially lower the pH of the solution to about 4.8. The pH rebounds to 5.7 after 5 hour. This observation suggests that the formation of new species may have some relationship with the pH of the solution.



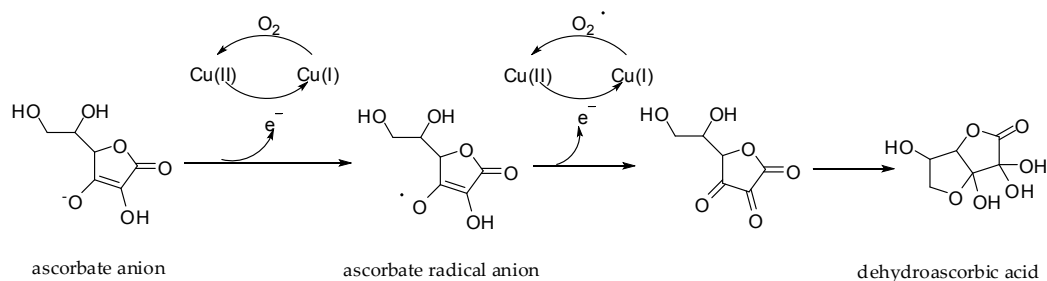
**Figure 13: UV-Vis spectrum of ATCUN-HH and Cu(II) in the presence of excess ascorbic acid.**

ATCUN-HH = 2.4 mM, Cu(II) = 2.4 mM, ascorbic acid = 8 mM, experiment was conducted in 50 mM HEPES buffer, pH=7.4. the pH of which shifted from 7.4 to 4.8 upon addition of ascorbic acid, then back up to 5.8 after 5 h.

### **3.2.6 Analysis of peptide-copper binding by the oxidation of ascorbic acid**

The aerobic oxidation of ascorbic acid is very slow when there are no catalytic metal ions present. However, when redox active metal ion such as Cu or Fe is present, ascorbic acid will be readily oxidized (Figure 14).[41] The coordination environment around metal complexes can significantly attenuate the rate of metal-catalyzed ascorbate oxidation. Chapter 2 described Cu(II) and Cu(I) binding properties for various histidine-

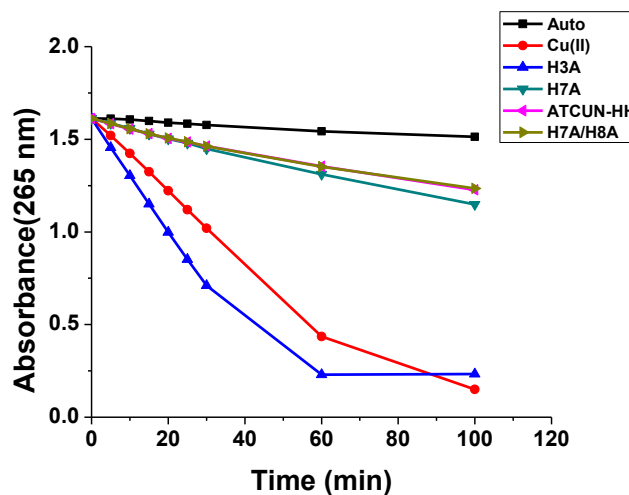
containing model peptides. The study of oxidation rate of ascorbic acid can provide an opportunity to integrate study the peptides-Cu binding properties.



**Figure 14: Copper catalyzed ascorbate oxidation. [34, 41]**

The catalytic reaction of ascorbic acid was studied by UV-Vis spectroscopy.

Ascorbic acid has an absorbance at 265 nm in 50 mM HEPES buffer, pH=7.4. In this experiment, ascorbic acid = 0.1 mM, CuSO<sub>4</sub> = 10 μM, peptide = 50 μM. The model peptides H3A, ATCUN-HH, H7A and H7A/H8A were tested in this experiment.



**Figure 15: UV-Vis absorbance changes at 265 nm of 0.1 mM ascorbic acid with different peptides and Cu(II) in aerobic conditions, pH=7.4.**

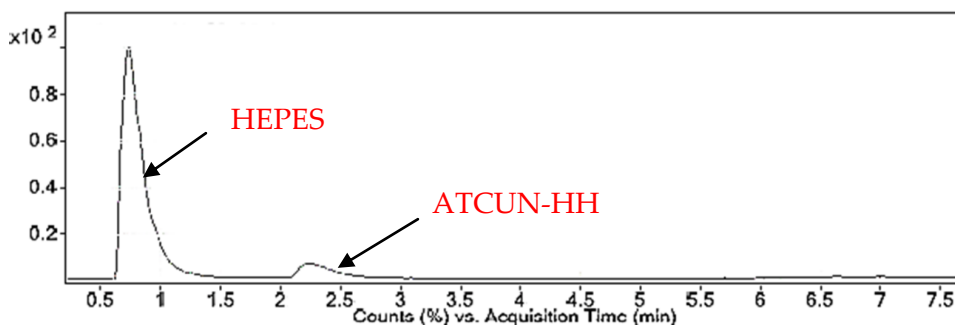
In Figure 15, auto-oxidation of ascorbic acid in pH 7.4 is very slow, as indicated by the minimal decrease of  $A_{265}$  over time. In contrast, free Cu(II) ion can robustly catalyze the oxidation of ascorbic acid, as indicated by the decrease in  $A_{265}$  over time. Except for H3A peptide, ATCUN-HH, H7A/H8A and H7A can suppress the rate of copper catalyzed ascorbic acid. This result indicates that these peptides bind Cu in a coordination environment that minimizes Cu redox-cycling between Cu(II) and Cu(I) oxidation states. Notably, all of these peptides contain an Cu(II)-ATCUN binding site. However, H3A seems to slightly increase the oxidation rate in this condition compared to free Cu(II), suggesting that H3A binds Cu in a manner that favors its redox-cycling between Cu(I) and Cu(II). H3A does not contain an ATCUN-Cu(II) binding site, but does have some affinity for Cu(I) with its His-His site.

### ***3.3 Mass spectral analysis of Cu-peptide-ascorbic acid reactions***

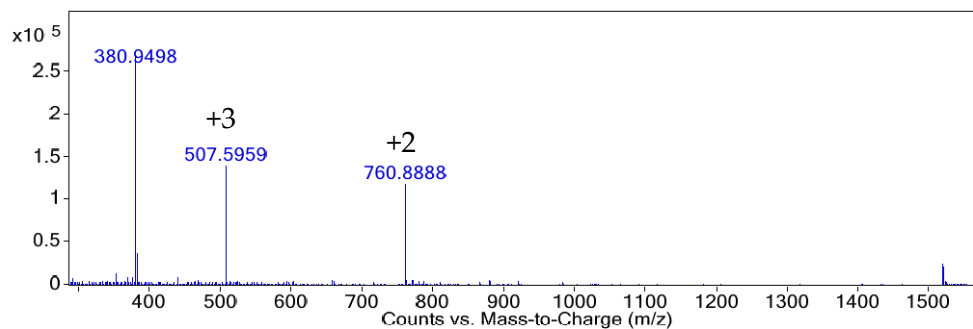
#### **3.3.1 LC-MS results**

In order to identify potential reaction products present in samples that show an absorbance band around 390 nm, LC-MS data were collected on samples of the Cu(II)/peptide solution and Cu(II)/peptide solution that had been incubated with ascorbic acid for 1 hour. Figure 16 A shows the total ion chromatogram (TIC) result for a Cu(II)-ATCUN-HH solution, which contains two observed peaks. The first peak that elutes close to the solvent front corresponds to the HEPES buffer. The peak eluting between 2-2.5 min provides an extracted ion chromatogram with m/z values of 760.8888,

507.5955 and 380.9498. These mass/charge values correspond to  $[P+2H]^{2+}$ ,  $[P+3H]^{3+}$  and  $[P+4H]^{4+}$ , where P stands for the peptide mass ion and H is an associated proton (Figure 16 B). The calculated mass of this species is 1519.78 Da which attributed to the free ATCUN-HH peptide, the theoretical mass of ATCUN-HH peptide is 1519.76 Da. Given the acidic nature of the chromatography elution conditions, it is not surprising to find apo peptide in the chromatogram, even from solutions that contained Cu initially.



A

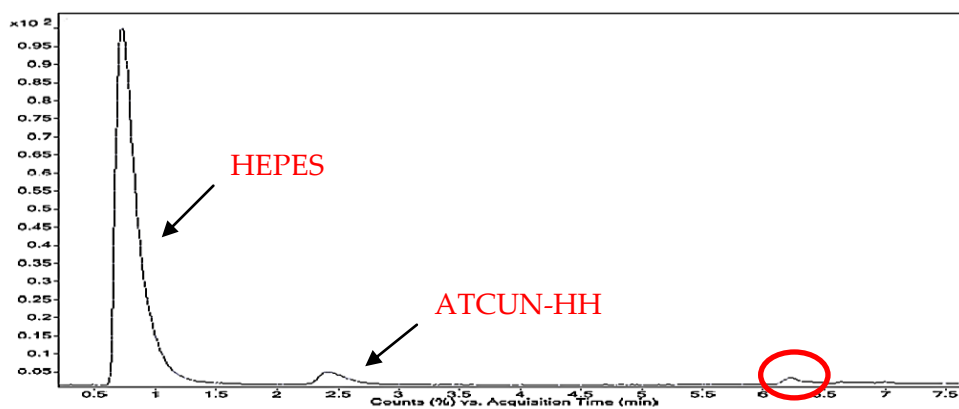


B

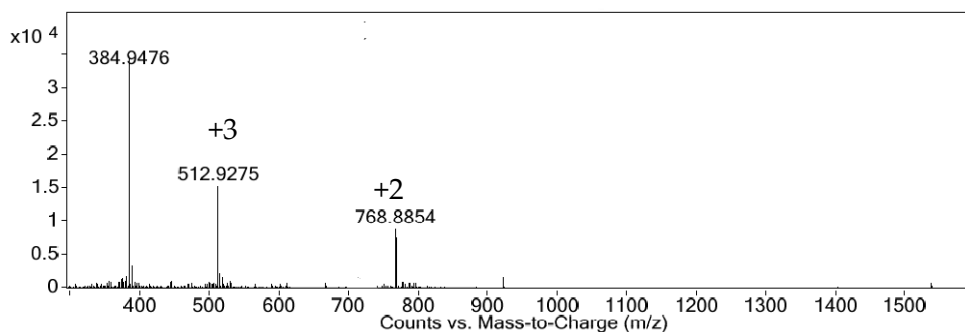
**Figure 16: LC-MS result of ATCUN-HH with Cu(II) in 50 mM HEPES buffer pH=7.4.**

ATCUN-HH = 0.3 mM, CuSO<sub>4</sub> = 0.3 mM. A) Total ion chromatogram resulting from a sample containing ATCUN-HH with Cu(II). B) Mass spectrum extracted from the TLC between 2-2.5 min, consistent with ATCUN-HH peptide.

Figure 17 reveals an addition peak in the chromatogram from samples incubated with ascorbic acid for 1 h. The new peak eluting at 6.2 min corresponds to  $m/z=768.8854$ , 512.9275 and 384.9476 (Figure 17 B). These values are consistent with a peptide of mass difference of  $\Delta m = +15.99$  Da compared to ATCUN-HH peptide. This result shows that solution conditions that lead to formation of the absorbing species with  $\lambda_{\max}$  390 nm also generate some fraction of oxidized peptide.



A



**Figure 17: LC-MS result for ATCUN-HH and Cu(II) in the presence of 1 mM ascorbic acid in 50 mM HEPES buffer pH=7.4.**

ATCUN-HH = 0.3 mM, Cu(II) = 0.3 mM. A) TIC of ATCUN-HH and Cu(II) with excess ascorbic acid. B) Extracted mass spectrum corresponding to the peak eluting at 6.2 min in spectrum A.

### 3.3.2 MALDI study

Matrix-assisted laser desorption/ionization (MALDI) is an ionization technique always used in the analysis of protein and peptide. Model peptide-Cu(II) solution in the presence of excess ascorbic acid were studied by MALDI.

**Table 3: MALDI result of ATCUN-HH with different concentration of Cu(II) in the presence of excess ascorbic acid.\***

	P	P+16	P+32	P+Cu	P+Cu+16	P+Cu+32	P+2Cu
0.5 eq	X			X			
0.6 eq	X			X			
0.8 eq	X	X		X			
1 eq	X	X	X	X	X		X
1.2 eq	X	X	X	X			
1.4 eq	X	X	X	X			
1.5 eq	X	X	X	X			
2 eq	X	X	X	X			

\*ATCUN-HH = 0.3 mM, Cu(II) concentration ranged from 0.5-2 equivalents, ascorbic acid = 1 mM, 50 mM HEPES buffer pH=7.4 has been used. After 1 hour MALDI test performed for all samples. The "X" in the table represent that the sample has this peak.

In previous experiments we studied the effect of the stoichiometry of peptide and Cu(II) on the formation of the new peak around 390 nm. It suggests that when the ratio of ATCUN-HH to Cu(II) is approximately 1, the addition of ascorbic acid can lead

to the formation of the 390 peak, while much more peptide or Cu(II) cannot generate this peak. In order to verify the differences between each ratio, after the UV-Vis spectroscopy study samples were collected for MALDI. Table 3 shows that the ATCUN-HH peptide is oxidized except when 0.5 equivalents and 0.6 equivalents of Cu(II) is present.

**Table 4: MALDI result for different peptide with Cu(II) and ascorbic acid\***

Cu+acid	P	P+16	P+32	P+Cu	P+Cu+16	P+Cu+32	P+2Cu
ATCUN-HH	X	X		X	X		X
H3A	X	X		X			
H7A/H8A	X			X			
Ctrl	X	X	X	X	X		X
F9AA	X	X		X	X		X

\* Peptide = 0.3 mM, CuSO<sub>4</sub> = 0.3 mM, ascorbic acid = 1 mM, experiments conducted in 50 mM HEPES buffer pH= 7.4. MALDI data collected after all the components mixed for 1 hour.

The effect of different reducing agent also been studied by MALDI. Table 4 shows that except for H7A/H8A all the model peptides have an oxidation peak after the addition of ascorbic acid for 1 hour. There are no significant differences between ATCUN-HH and the other peptides. In Table 5 the addition of sodium dithionite after 1 hour also leads to the oxidation peak formation for the model peptides. It is important to note that for the H7A/H8A peptide, neither ascorbic acid nor sodium dithionite can oxidize the peptide after 1 hour. Previous studies show that only ATCUN-HH and

F9AA have the absorbance near 390 nm with Cu(II) and excess ascorbic acid. However, this new peak is specific for ascorbic acid. In this experiment the addition of ascorbic acid or sodium dithionite can oxidize all of the model peptides. MALDI analysis shows that the addition of ascorbic acid or sodium dithionite does not have lots differences for all the model peptides. It is unknown if ascorbic acid and sodium dithionite oxidize peptides via the same pathway.

**Table 5: MALDI result for different peptide with Cu(II) and sodium dithionite\***

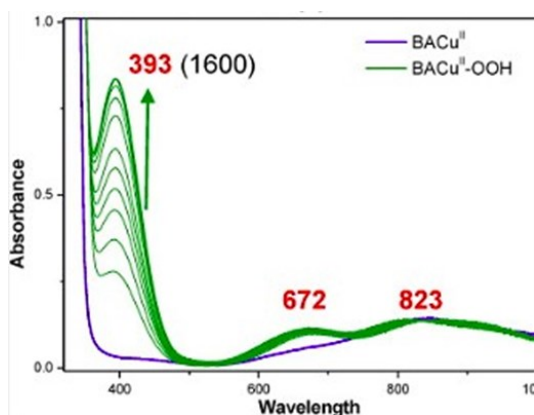
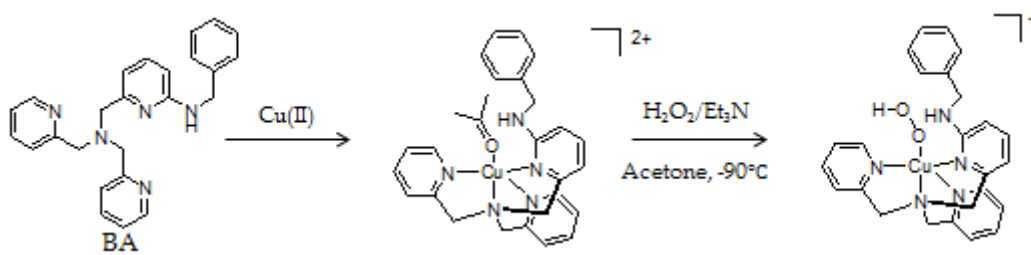
Cu+Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	P	P+16	P+32	P+Cu	P+Cu+16	P+Cu+32	P+2Cu
ATCUN-HH	X	X	X	X	X	X	
H3A	X	X	X	X			
H7A/H8A	X			X			
Ctrl	X	X	X	X	X		
F9AA	X			X			

\* Peptide = 0.3 mM, CuSO<sub>4</sub> = 0.3 mM, sodium dithionite = 1 mM, experiments conducted in 50 mM HEPES buffer pH= 7.4. MALDI data collected after all the components mixed for 1 hour.

### 3.4 Summary

In this chapter, UV-Vis spectroscopy and mass spectroscopy have been utilized to study the new species that is formed when excess ascorbic acid is added to a Cu(II)-ATCUN-HH solution. Based on these studies, several conclusions can be drawn. First,

the formation of the new species requires the presence of oxygen or another oxidizing agent such as hydrogen peroxide. Secondly, the stoichiometry of the ATCUN-HH peptide and Cu(II) can affect the formation of new species. When the ratio of peptide to Cu(II) is around 1 the peak at 390 nm can form. Third, it seems that the formation of the new species requires the presence of ascorbic acid.



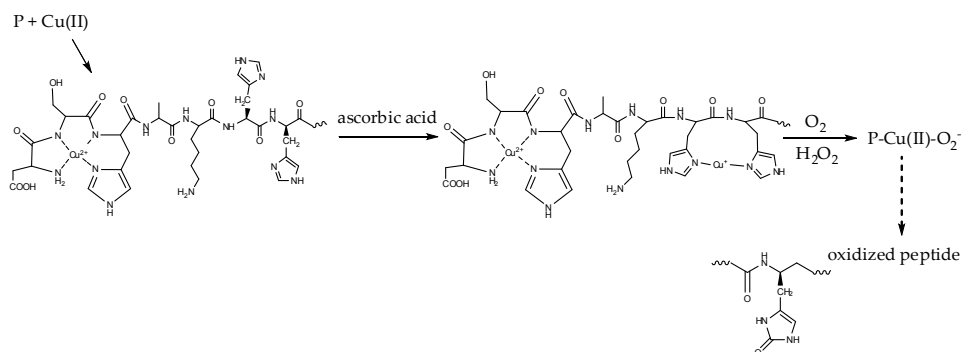
**Figure 18: A) Structure of BA.[42] B) UV-Vis spectrum of the generation of BACu(II)-OOH.[42]**

An early report suggested that a short metal binding fragment of amyloid peptides has catechol- and phenol- oxidase like activity.[43-45] This oxidative activity

includes oxygen binding and form reactive intermediate by a di-Cu(II) center.[43-46] One study shows that Cu(II)-histatin 5 can oxidize catechol, showing enzyme-like kinetics.[45] Based on these precedents, the new peak near 390 nm may be related to copper-peroxo or copper-hydroperoxo species which are regarded as reactive intermediates in biological oxygenases such as dopamine  $\beta$ -hydroxylase.[46] Previous study shows that the synthesized ligand named BA can form a complex with Cu(II) (Figure 18A).[42] This study shows that Cu(II)-hydroperoxo species can be produced by the reaction of this copper (II) complex and H<sub>2</sub>O<sub>2</sub> in the presence of a base (Figure 18A).[42] This Cu(II)-hydroperoxo complex has a LMCT band at 350-400 nm which is quite similar to the new peak in our experiment (Figure 18B).[42] In addition, the LC-MS and MALDI results show that the peptide has been oxidized after adding ascorbic acid to the Cu(II)-peptide solution.

Figure 19 shows the hypothesis of the formation of the new species which has absorbance at 390 nm. Previous studies have shown that Cu(II)-histatin peptides with ascorbic acid can produce H<sub>2</sub>O<sub>2</sub>, which may facilitate the formation of Cu-hydroperoxo species.[40] It is possible that with O<sub>2</sub> and ascorbic acid present, the Cu-ATCUN-HH complex rapidly forms Cu(I) species and then is oxidized to the metal-peroxo or metal-hydroperoxo species (Figure 19). These intermediate may have absorbance near 390 nm, and finally lead to the oxidation of peptides. From the mass spectroscopy result it is

possible that the histidine residues in the sequence are oxidized. Further study is needed to elucidate the new peak formed in these experiments.



**Figure 19: Hypothesis of the formation of new species which has absorbance at 390 nm.**

P represents ATCUN-HH or F9AA peptide. After the addition of excess ascorbic acid, peptide-Cu(II) may form a Cu-peroxo complex and followed by peptide oxidation.

### 3.5 Material and methods

#### Material

L-ascorbic acid, sodium dithionite and HEPES are all from Sigma-Aldrich. A 0.05 M HPEES buffer solution was prepared by dissolving HEPES in Nanopure water. The pH of this solution is adjusted by 0.1 M NaOH.

#### UV-Vis spectroscopy

Absorption spectra were collected by either a Cary50 UV-Vis spectrophotometer or an SI Photonics (Tucson, AZ) model 420 fiber optic CCD array UV-Vis spectrophotometer.

#### Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) were collected on an Agilent 1100 Series spectrometer. The condition of the LC-MS is on a C-18 column with a linear 15 minutes gradient from 0-60% acetonitrile in water with 0.1% TFA.

### **MALDI study**

A saturated matrix solution was made by dissolving  $\alpha$ -Cyano-4-hydroxycinnamic acid in ethanol. The final samples achieved by add 9  $\mu$ L matrix and 1  $\mu$ L sample solution, 1  $\mu$ L of this mixture is applied to a stainless-steel sample well. Samples were measured by bruker Daltonics Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA).

## **4. Study of reactive oxygen species in vitro**

### **4.1 Background and significance**

The term “ Reactive oxygen species” (ROS) is used for oxygen species which more reactive than triplet oxygen.[47] The production of ROS such as superoxide, hydrogen peroxide and the hydroxyl radical are always related with metal ions such as Fe(II,III) and Cu(I, II).[47] ROS are often produced as unwanted byproducts of cellular respiration. ROS production is a source of oxidative stress and loss of the ability to control ROS results in oxidative damage which is related to many diseases.[47]

Understanding of the mechanism of antifungal activity of histatin 5 is important if histatins are to be considered as potential candidates for drug therapy. It is know that the antifungal activity of histatin 5 is limited to cells that undergo cellular respiration. Since respiratory disorders can lead to the production of ROS, it is possible that these oxidative species may play a role in the antimicrobial activity of histatin5.[20]

Moreover, previous studies about the in vitro production of ROS by histatins and copper suggest that high levels of hydrogen peroxide can be generated in a solution containing Cu(II), histatin 5 or histatin 8 and a reductant, either ascorbic acid or cysteine.[40] It shows that the amount of superoxide produced when histatin 5 or histatin 8 is present is greater than when there is no peptide present in the solution.[40] Therefore, when Cu(II) and histatin 5 and a reductant are present in solution, peroxide and superoxide can be produced. Considering that the new species discussed in chapter

3 may be related to ROS formation, the DCF (2',7'-dichlorofluorescein) assay is used to study ROS formation by histatin model peptides in this chapter.

## 4.2 Results

### 4.2.1 Introduction of DCF assay

DCFDA is one of the most popular probes used to detect ROS.[48] DCFDA is deacetylated by NaOH to yield H<sub>2</sub>DCF (dihydrodichlorofluorescein), H<sub>2</sub>DCF is then added to a 96-well microtiter plate. The oxidation of this molecule to the fluorochrome DCF results in fluorescence.[47, 48] The fluorescence turn on indicates the presence of a species capable of 2-electron oxidation, which can include ROS.[47]

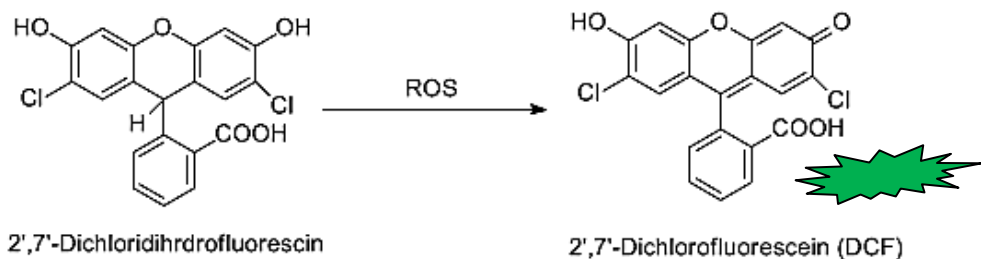


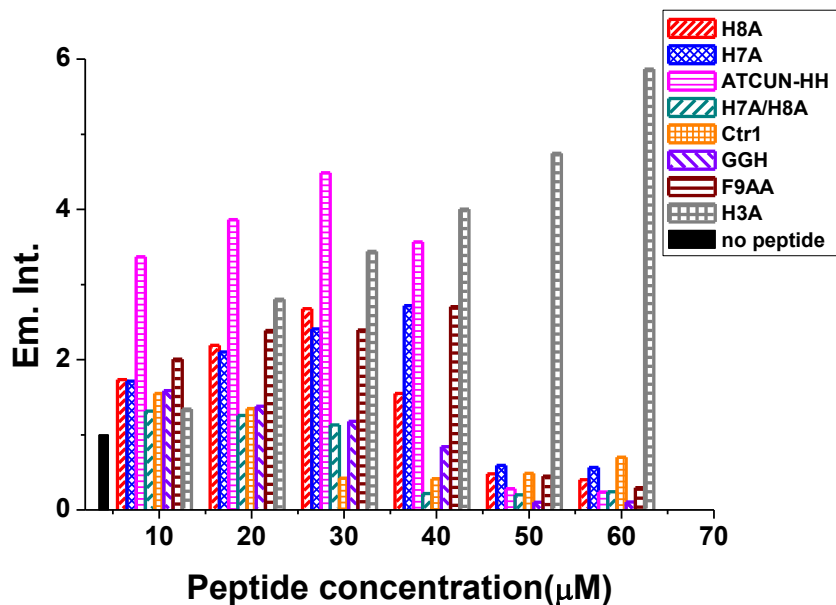
Figure 20: Mechanism of DCF assay.

### 4.2.2 Comparison of histatin model peptides

It has been shown previously that ATCUN-HH, Cu(II) and ascorbic acid can generate a new species that has an absorbance band at 390 nm. However, this feature only occurred for the ATCUN-HH and F9AA peptides. In order to elucidate whether this new species is related to ROS formation and how the sequence of these peptides affects their redox activity, the DCF assay was performed for ATCUN-HH, H7A, H8A,

H7A/H8A, H3A, F9AA, Ctr1 and GlyGlyHis peptide, a short ATCUN peptide that can bind Cu(II).

The Cu-catalyzed aerobic oxidation of ascorbic acid generates ROS that are capable of oxidizing H<sub>2</sub>DCF to fluorescent DCF. In this experiment, 40  $\mu$ M CuSO<sub>4</sub>, 1 mM ascorbic acid and 10  $\mu$ M H<sub>2</sub>DCF were incubated at room temperature with 10-60  $\mu$ M concentration of each peptide and the fluorescence intensity was measured after 2 hours. As a control, a well that only contained Cu(II), ascorbic acid and H<sub>2</sub>DCF was also measured.



**Figure 21: Fluorescence intensity of DCF recorded 2 h after reacting model peptides, Cu(II) and H<sub>2</sub>DCF in the presence of excess ascorbic acid.**

In each well, Cu(II)=40  $\mu$ M, ascorbic acid=1 mM, the concentration for ATCUN-HH, Ctr1, GlyGlyHis, H7A/H8A, H7A, H8A, F9AA, H3A is 10, 20, 30, 40, 50, 60  $\mu$ M in 50 mM pH 7.4 HEPES buffer. Fluorescence intensity measured after 2 hour.

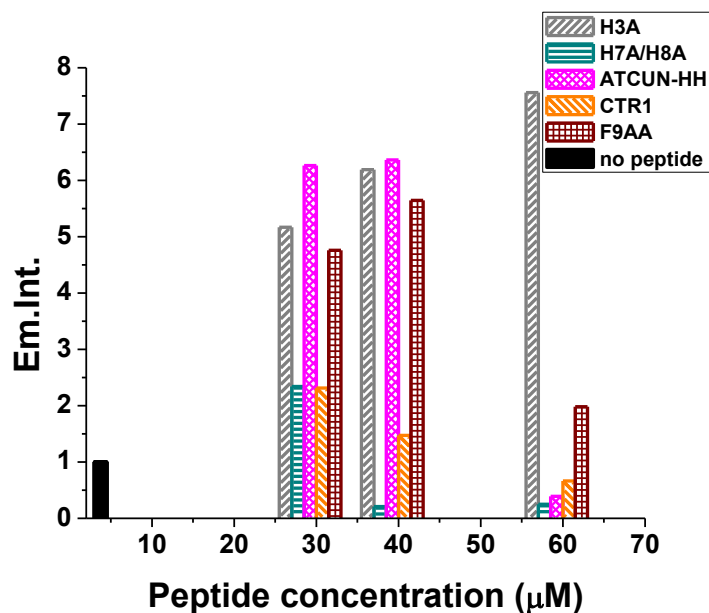
Figure 21 shows the result of this experiment. In order to acquire a clear understanding of the results we set the intensity of the control well to 1. A bar with intensity greater than 1 indicates that those conditions that favor DCF oxidation over and above the background condition of Cu(II) alone, while an intensity less than one indicates that the peptide inhibits the copper-catalyzed reactions that lead to DCF fluorescence. Most of the peptides favored greater DCF formation than the control at substoichiometric concentrations with respect to Cu, then became inhibitory when the ratio of peptide : Cu exceed 1. When the peptide concentration is less than 40  $\mu\text{M}$ , ATCUN-HH peptide dramatically increases the fluorescence intensity compared to when no peptide is present and compared to the other peptide. It is worth noting that the ratio of ATCUN-HH to Cu(II) can affect the fluorescence intensity. When ATCUN-HH/Cu(II) is greater than 1, ATCUN-HH inhibits DCF fluorescence intensity. Previous studies in chapter 3 shows that the ratio of ATCUN-HH to Cu(II) can affect the absorbance of the new 390 nm species. This indicates that the new species may be related to ROS formation.

The GlyGlyHis peptide, which contains only an ATCUN motif, inhibits DCF fluorescence at all concentration tested. It is notable that the Ctr1 peptide, which contains both an ATCUN and a His-His motif, also inhibits at all concentrations tested. In contrast, H3A which lack an ATCUN-Cu(II) site but does contain a His-His motif increases that ROS formation in all the conditions tested. This latter result is consistent

with the faster rate of ascorbate oxidation observed for H3A compared to Cu alone or Cu with the other peptides (Figure 15).

### 4.2.3 Comparison of different reducing agents

In order to verify the functions of different reducing agents, sodium dithionite was tried in the DCF assay. In this experiment, 40  $\mu\text{M}$  Cu(II), 1 mM sodium dithionite and 10  $\mu\text{M}$  H<sub>2</sub>DCF were combined in each well. For each peptide, the concentration ranged from 30-60  $\mu\text{M}$  and the fluorescence intensity was measured after 2 hours. A control well that contained Cu(II), sodium dithionite and H<sub>2</sub>DCF was also measured.



**Figure 22: Fluorescence intensity of DCF recorded 2 h after reacting model peptides, Cu(II) and H<sub>2</sub>DCF in the presence of excess sodium dithionite.**

In each well the Cu(II)=40  $\mu\text{M}$ , sodium dithionite=1 mM, the concentration for ATCUN-HH, Ctr1, F9AA, H3A, H7A/H8A is 30, 40, 60  $\mu\text{M}$ , 50 mM pH 7.4 HEPES buffer has been used. Fluorescence intensity collected by plate reader after 2 hours.

Figure 22 shows that the addition of H7A/H8A and Ctr1 peptide cannot increase the fluorescence intensity. When the ratio of peptide/Cu(II) is greater than 1, the addition of the ATCUN-HH peptide also cannot increase the fluorescence intensity. However, when peptide/Cu(II) is less than 1, the addition of ATCUN-HH can significantly increase the fluorescence intensity. This is similar to the results obtained when ascorbic acid is present. It is interesting that the addition of H3A can increase the fluorescence in all conditions which is similar to the results obtained using excess ascorbic acid. Therefore, in the presence of either reducing agent ATCUN-HH peptide promotes the Cu-catalyzed oxidation that lead to increasing the DCF fluorescence intensity. This indicates that the addition of ATCUN-HH generates more ROS than when no peptide is present. However, previous UV-Vis spectroscopy studies show that the addition of sodium dithionite to a Cu(II)-ATCUN-HH solution cannot generate the new peak near 390 nm. Based on these results, it is not clear whether the new species which has absorbance near 390 nm is related to the formation of ROS.

### **4.3 Summary**

Previous studies show that the antifungal activity of histatin 5 may be related to ROS formation.[20] In this chapter, we studied ROS formation by the DCF assay. This study has demonstrated that DCF fluorescence is most elevated when aerobic mixtures of Cu and ascorbic acid are mixed with ATCUN-HH, F9AA, and H3A peptides, with some variation in optimal peptide : Cu ratios. On the other hand, the reaction is

inhibited by GlyGlyHis, Ctr1 and H7A/H8A peptides. It may be relevant to note that none of the inhibitory peptides generate a 390 nm absorbance peak when mixed with Cu(II) and ascorbic acid, whereas 2 of 3 the stimulatory peptides do. It is interesting to speculate whether the species giving rise to the 390 feature may also be responsible for H<sub>2</sub>DCF oxidation. The H3A result contradicts this hypothesis, since this peptide does not support formation of the 390 species. However, H3A turns over ascorbic acid much more efficiently than either ATCUN-HH or F9AA, both of which retard the Cu-catalyzed oxidation of ascorbic acid. These differences in reactivity between H3A and both ATCUN-HH peptides suggest that their mechanisms for H<sub>2</sub>DCF oxidation may be different. For example, by promoting Cu(II)/Cu(I) cycling and ascorbic oxidation but not trapping Cu(II), H3A may promote formation of freely diffusible ROS species that proceed to oxidize H<sub>2</sub>DCF, whereas ATCUN-HH could generate a reactive Cu-bound ROS intermediate that could react with H<sub>2</sub>DCF. Either way, "ROS" are efficiently produced by the histatin-related peptides, Cu(II) and a reducing agent in vitro under near physiological conditions. It remains to be seen whether these species are responsible for ROS generation in vivo.

In general, some strongly coordinating ligands such as chelating agents and some peptides can inhibit metal-promoted ROS generation.[49, 50] However, other proteins such as amyloid  $\beta$ [51, 52] and  $\alpha$ -synuclein that bind copper can still produce ROS.[53] It seems that the ATCUN-HH-Cu(II) system is in the latter class. Our DCF

assay confirmed that in the presence of the ATCUN-HH peptide there is a large amount of ROS formed compared to when no peptide is present. This is consistent with previous studies. However, the coordinating ability of ascorbic acid and sodium dithionite indicates that complex such as Cu(peptide)(ascorbate) or Cu(peptide)(sodium dithionite) could be involved.

However, there are several aspects of these studies that require further study. First, the ratio of ATCUN-HH to Cu(II) can affect the ROS formation. Previous studies show that when ATCUN-HH is in excess the absorbance at 390 nm does not form as a distinct peak after addition of ascorbic acid, although non-specific changes in the absorbance spectrum indicate molecular alternations have taken place that change the spectrum. It is possible that the 390 nm species forms fleetingly before extensive oxidative modification occurs. Secondly, it would be important to study if ROS formation produced via the same pathway regardless of which reducing agent is used.

#### ***4.4 Materials and methods***

##### **General materials**

L-ascorbic acid, sodium dithionite, 2,7-dichlorofluorescein diacetate and HEPES are all from Sigma-Aldrich.

H<sub>2</sub>DCFDA was dissolved in ethanol to give a 10 mM stock solution, this solution was then dilute to 1 mM by HEPES buffer (pH 7.4). 2 mL of 0.01 M NaOH was added to 0.5 mL H<sub>2</sub>DCFDA solution and after 30 min 10 mL of 50 mM HPPES buffer was added

to get a 40  $\mu\text{M}$  stock solution. This stock solution stored in fridge and prepared freshly each day.[54]

#### **DCF assay**

In a typical set of experiments using a 96-well assay plate, 50  $\mu\text{L}$  peptide-Cu(II) solution was first added to a well. To start the reaction 50  $\mu\text{L}$  of 2 mM ascorbic acid (or sodium dithionite) and 20 mM  $\text{H}_2\text{DCF}$  solution in HEPES buffer was added. The fluorescence intensity of the well(s) was then recorded on a Perkin Elmer Victor 1420 plate reader under room temperature. Shaking performed before measuring.

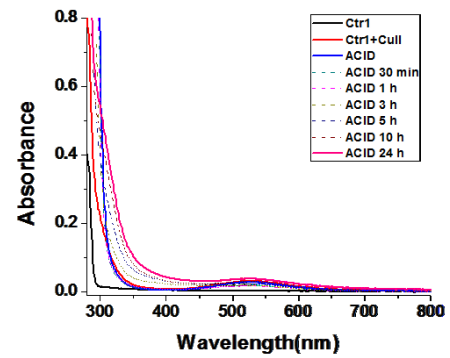
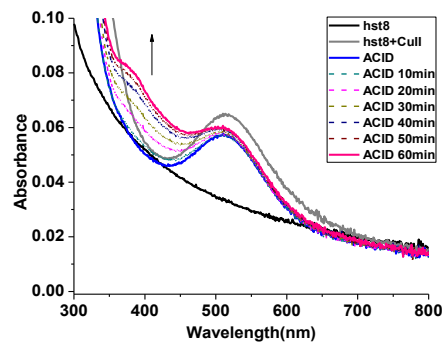
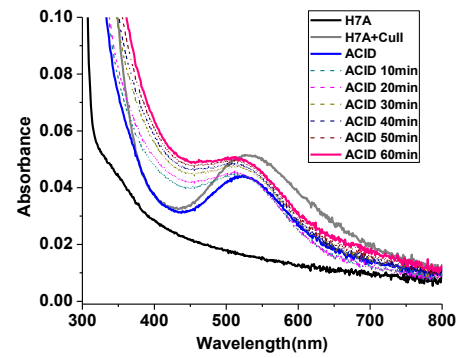
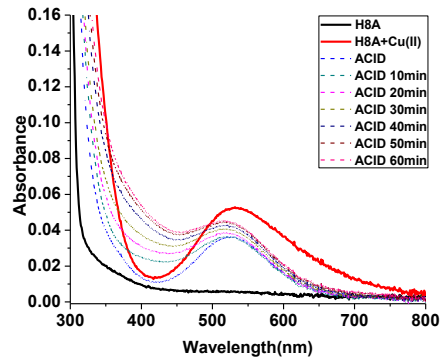
## 5. Conclusion

The exact mechanism of the antifungal activity of histatin 5 is still under debate. Some studies proposed that copper binding property may play a significant role in the performance of antifungal activity of histatin 5.[29, 30] The present study confirmed that synthetic histatin 5 model peptide ATCUN-HH can bind Cu(II) at the ATCUN motif. The Cu(I) binding properties of model peptides have been studied by Fz competition assay, which indicates ATCUN-HH has significant affinity for Cu(I) via histidine residues. These results imply the flexibility of histatin 5 in binding Cu(II) and Cu(I)

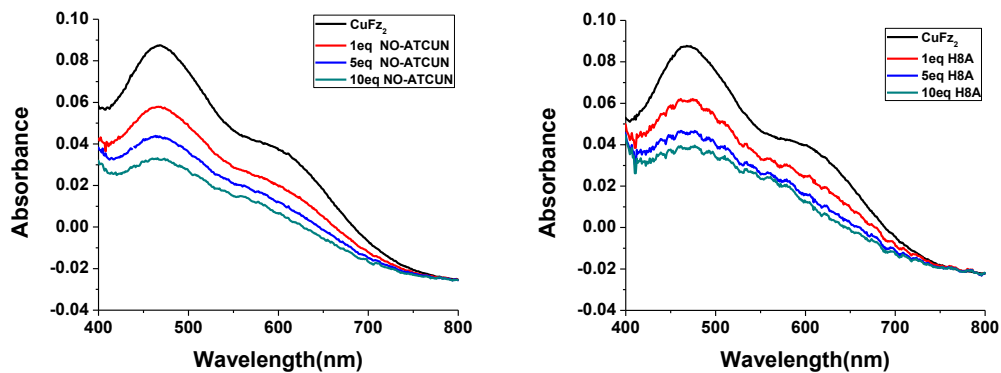
It is worth noting that a new species forms when excess ascorbic acid is present in the ATCUN-HH-Cu(II) solution. Our study shows that the formation of the new species requires the presence of oxidizing agents and ascorbic acid. The approximate 1:1 ratio of peptide to Cu(II) is also critical for the generation of the new species. Moreover, DCF study suggests that in the presence of reducing agents, ATCUN-HH with Cu(II) can increase ROS formation compare to no peptide present. The current results suggest that the antimicrobial activity of histatin 5 may derive from its metal binding property and oxidative activity. Further studies are needed to investigate the nature of the new species. In addition, the whole sequence of histatin 5, rather than merely the ATCUN-HH fragment should be explored for future mechanism investigation.

# Appendix A

A.1 Peptides with 1 equivalent  $\text{CuSO}_4$  (0.3 mM) in the presence of excess ascorbic acid (1 mM) in 50 mM HEPES pH=7.4.

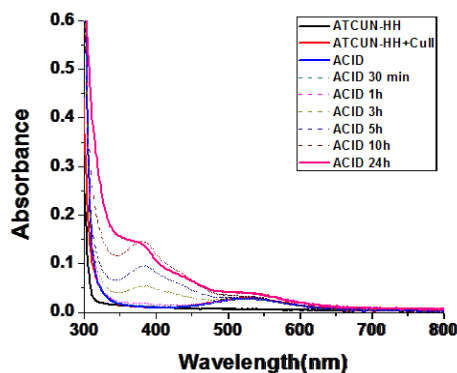


A.2 Cu(I) binding study. Cu(I) concentration 20  $\mu\text{M}$ , Fz concentration 50  $\mu\text{M}$ , add 1, 5, 10 equivalents peptide, 50 mM HEPES buffer pH=7.4. Experiment was conducted in nitrogen-filled glove box.

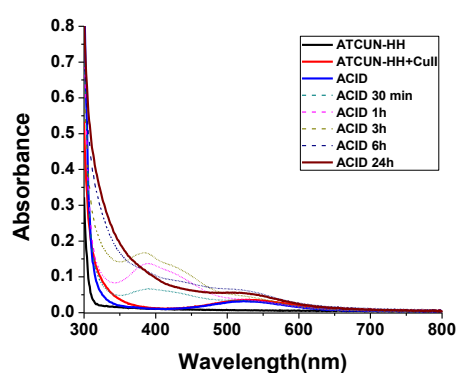


A.3 Change the ratio of Cu(II) to ATCUN-HH. ATCUN-HH=0.3 mM, ascorbic acid =1 mM, 50 mM HEPES buffer, pH=7.4. Cu(II) concentration ranged from 0.8-1.5 equivalents of ATCUN-HH.

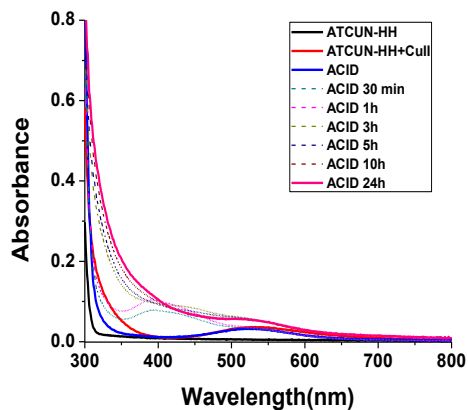
0.8 eq



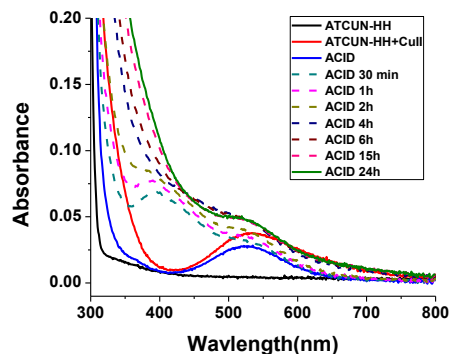
1.2 eq



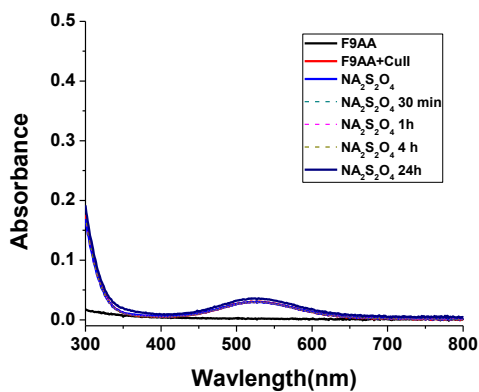
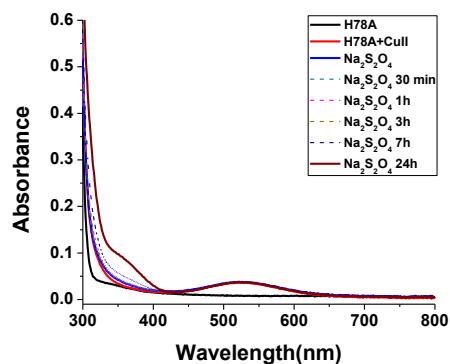
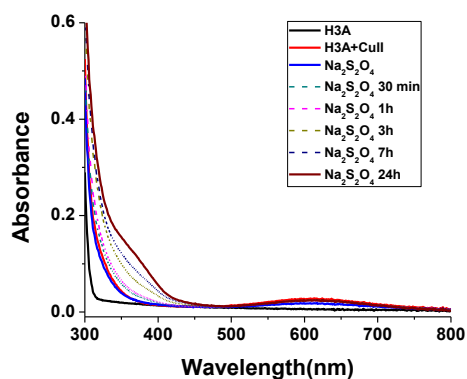
1.4 eq



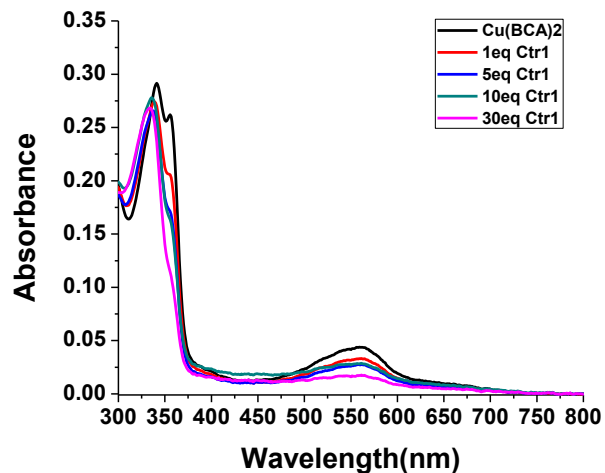
1.5 eq



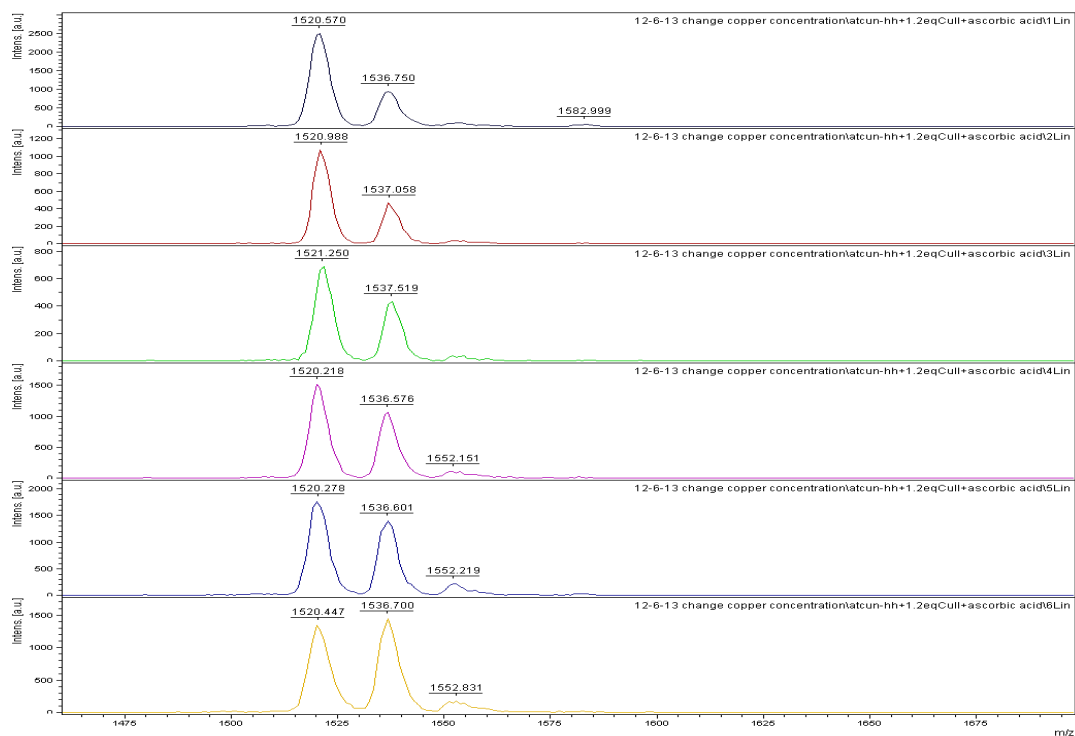
A.4 The effect of different reducing agent. Peptide (0.3 mM) and Cu(II) (0.3 mM) in the presence of 1 mM sodium dithionite, 50 mM HEPES buffer pH=7.4.



### A.5 Ctr1 titrate Cu(I)(BCA)<sub>2</sub>.



A.6 MALDI result of ATCUN-HH with 1.2 equivalents of Cu(II) in the presence of excess ascorbic acid. MALDI test performed for sample collected at 1 h, 2 h, 5 h, 7 h, 10 h, 24 h.



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