Copper and Iron Complexes of Linear and Crosslinked Polymers as Catalysts for
Phosphoester Hydrolysis and Oxidative Transformation of
Phenolic and Catecholic Substrates

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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# Table of Contents

List of Tables iv

List of Figures vii

List of Abbreviations xiii

Abstract xiv

CHAPTER I. INTRODUCTION 1

I. Metallopolymer 1

II. Classification of metal complexes and metals in macromolecules 4

III. Metal containing biomacromolecules 7

IV. Polymer based heterogeneous catalysis 8

V. Linear and crosslinked copolymers and their metal complexes 11

VI. Advantages and disadvantages in use of polymeric catalysts 12

VII. List of References 4

CHAPTER 2. SYNTHESIS AND METAL BINDING OF LINEAR COPOLYMERS (VINYL PYRIDINE-co-X) PREPARED VIA FREE RADICAL POLYMERIZATION (X = Acrylamide, Methylmethacrylate, styrene, acrylic acid) prepared via free radical polymerization (X = acrylamide, methylmethacrylate, styrene, acrylic acid)

I. Introduction 16

1. Free radical polymerization 16

2. Copolymerization 23

II. Experimental 27

1. Monomers and initiator used in polymerization 27

2. Copolymer synthesis 29

a. Vinylpyridine-co-acrylamide series 29

b. Vinylpyridine-co-acrylic acid series 33

c. Preparation of vinylpyridine-co-styrene and vinylpyridine-co- methylmethacrylate series 36

d. Product yields 37

III. Results and Discussion 41

A. Polymer characterization 41

1. Repeating unit determination using $^1$HNMR 41
2. Functional group determination using $^{13}$CNMR and HMQC

B. Transition metal binding to synthesized polymers

1. Metal binding stoichiometry by use of Co(II) as a probe

2. IR and UV−vis of synthesized polymers and their metal derivatives

IV. Conclusion and Perspectives

V. References

CHAPTER 3. HYDROLYTIC ACTIVITIES OF Fe(III) AND Cu(II) DERIVATIVES OF SYNTHESIZED COPOLYMERS AND COMMERCIAL AVAILABLE RESINS TOWARDS PHOSPHOESTER HYDROLYSIS

I. Introduction

II. Experimental: Materials and Methods

III. Results

1. Hydrolytic activity of Fe$^{3+}$- P1
   A. Metal binding and catalyst stoichiometry
   B. Kinetic characterization of phosphoester hydrolyses
   C. Effect of pH on BNPP hydrolysis
   D. Catalyst recycling
   E. Molecular mechanics calculations
   F. Polymer design
   G. Inhibition of BNPP hydrolysis by HPO$_4^{2−}$
   H. Oxidative plasmid DNA cleavage

2. Heterogeneous hydrolysis catalyzed by metal complexes of crosslinked resins

IV. Concluding Remarks and Future Perspectives

CHAPTER 4. OXIDATION AND HYDROXYLATION ACTIVITIES OF Cu(II) COMPLEXES OF SYNTHESIZED COPOLYMERS TOWARDS (POLY)PHENOLS

I. Introduction

1. Model systems for oxygen activation
2. Hydroxylation of phenolic substrates
3. Polymer−based systems in copper−centered oxidations
4. Applications in detoxification of pollutants
5. Reactive oxygen species (ROS), oxidative DNA cleavage and disease
6. Natural Antioxidants as Inhibitors

II. Experimental

1. Materials

   ii
2. Methods
   A. Spectrophotometric determination of rates of oxidative reactions 144
   B. DNA cleavage assays 151
3. Pre-equilibrium kinetics and catalytic constants 152
4. Bisubstrate reaction mechanism 154
5. Inhibition studies 157
III. Results and Discussion 159
   1. Polyphenol Oxidation 159
   2. Metal interactions in the dicopper center and its role in oxidative reactions 173
   3. Influence of azide in oxidative reactions 177
   4. Oxidation of neurotransmitters 183
   5. Inhibition of polyphenol oxidation 193
   6. Oxidative Plasmid DNA Cleavage 198
   7. Molecular Mechanics Calculations and Mechanism 202
   8. Hydroxylation of phenolic substrates by Cu(II)-P1 207
   9. Effect of dilution of Cu\textsuperscript{II} with Zn\textsuperscript{II} in hydroxylation reactions 216
   10. Kinetic Isotope Effect 217
   11. Mechanistic Job 218
   12. Effect of Sodium Borate 222
   13. Effect of pH on hydroxylation reaction 224
   14. Molecular Mechanics Calculations for phenol binding to Cu(II)–P1 225
   15. Catalytic mechanism for hydroxylation of phenolic substrates 227
   16. EPR characterization of the dinuclear center 229
   17. Natural antioxidants and inhibition studies 232
   18. Oxidative dechlorination of polychlorinated aromatic compounds 234
IV. Concluding Remarks and Future Perspectives 241
V. References 244

ABOUT THE AUTHOR End page
List of Tables

Table 2.1 Half life of AIBN as function of reaction temperature.  
Table 2.2 Value of Q and e parameters for monomers used in copolymer Synthesis  
Table 2.3 Calculated repeating units of synthesized copolymers  
Table 2.4 Physical properties of monomers used in polymerization  
Table 2.5 Mole ratios, monomer and initiator weights used in synthesis of Vp−co−Am 25:75% copolymer series  
Table 2.6 Mole ratios and monomer and initiator weights used in synthesis of the 4Vp−co−Am 50:50% copolymer series  
Table 2.7 Mole ratios and monomer and initiator weights used in synthesis of the 4Vp−co−Am 75:25% copolymer series  
Table 2.8 Mole ratios and monomer and initiator weights used in synthesis of the 4Vp−co−Aa 25:75% copolymer series  
Table 2.9 Mole ratios and monomer and initiator weights used in synthesis of the 4Vp−co−Aa 50:50% copolymer series  
Table 2.10 Mole ratios and monomer and initiator weights used in synthesis of the 4Vp−co−Aa 75:25% copolymer series  
Table 2.11 Molar ratios and monomer and initiator weights used in synthesis of the 4Vp−co−Mma and 4Vp−styrene 25:75% copolymer series  
Table 2.12 Product yields in polymerization of 4Vp:Am copolymers  
Table 2.13 Product yields in polymerization of 2Vp:Am copolymers  
Table 2.14 Product yields in polymerization of 4Vp:Aa copolymers
Table 4.9 Calculated apparent and intrinsic dissociation constants of neurotransmitters in presence of O$_2$ and in H$_2$O$_2$ 192

Table 4.10 Kinetic parameters for hydroxylation of phenolic substrates in presence of O$_2$ and in H$_2$O$_2$ 215
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Polymerization of vinylferrocene</td>
<td>1</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Mechanism of electron transfer in conducting polymers</td>
<td>3</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Metals bound at macromolecules</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Ligand of a metal complex as part of a macromolecule</td>
<td>5</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Physical incorporation of metal nanoparticles or metal complexes</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Possible arrangement of functional groups in synthetic and natural polymers</td>
<td>10</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Individual steps in free radical polymerization</td>
<td>18</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Decomposition of $\alpha,\alpha\text{-azobis(isobutynitrile)}$ (AIBN) to the dimethylcyan free radical</td>
<td>20</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Polymer structural elements</td>
<td>22</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Structure of monomers used in synthesis of copolymers</td>
<td>27</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>$^1\text{H}$ NMR spectra of 4Vp:Am (P1) in $d_6$-DMSO</td>
<td>43</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Example of average repeating unit in copolymer P1</td>
<td>44</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>$^1\text{HNMR}$ spectra of 4Vp: styrene in $d_6$-DMSO</td>
<td>46</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>$^1\text{HNMR}$ spectra of 4Vp:Mma and 4Vp:Aa in $d_6$-DMSO</td>
<td>47</td>
</tr>
<tr>
<td>Figure 2.9</td>
<td>$^1\text{HNMR}$ spectra of 2Vp:Am $d_6$-DMSO before and after the addition of D$_2$O</td>
<td>48</td>
</tr>
<tr>
<td>Figure 2.10</td>
<td>HMQC spectra of P1 in $d_6$-DMSO</td>
<td>51</td>
</tr>
<tr>
<td>Figure 2.11</td>
<td>$^{13}$CNMR spectra of P1 in d$_6$-DMSO</td>
<td>52</td>
</tr>
<tr>
<td>Figure 2.12</td>
<td>COSY spectra of P1 in d$_6$-DMSO</td>
<td>53</td>
</tr>
<tr>
<td>Figure 2.13</td>
<td>Paramagnetically shifted pyridine signals observed upon addition of two equivalents of Co(II) to P1 in d$_6$-DMSO</td>
<td>55</td>
</tr>
<tr>
<td>Figure 2.14</td>
<td>$^1$HNMR spectra of P1 before addition of 2.0 equivalents of Co(II) in d$_6$-DMSO</td>
<td>56</td>
</tr>
<tr>
<td>Figure 2.15</td>
<td>Diamagnetic region of $^1$HNMR spectra of P$_1$ in d$_6$-DMSO after addition of 2.0 equivalents of Co(II), in d$_6$-DMSO</td>
<td>57</td>
</tr>
<tr>
<td>Figure 2.16</td>
<td>IR spectra of P1 and Fe(III)-P1 in KBr</td>
<td>59</td>
</tr>
<tr>
<td>Figure 2.17</td>
<td>UV−Vis spectra of polymer during Fe(III) titration</td>
<td>60</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Hydrolysis of peptide and phosphoester bonds</td>
<td>67</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Proposed mechanism for phosphoester hydrolysis by kidney bean PAP</td>
<td>70</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Titration of Fe(III) into 1.0 mM RU P1 while monitoring hydrolytic activity</td>
<td>77</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Activity Job plot for Fe(III)−P1</td>
<td>79</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Initial rate of hydrolysis for several phosphoesters catalyzed by Fe(III)−P$_1$</td>
<td>81</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Effect of pH on hydrolysis of BNPP catalyzed by Fe(III)−P1</td>
<td>85</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Initial rate of hydrolysis for several phosphoesters catalyzed Fe(III)−P$_1$</td>
<td>87</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Recycling of catalyst monitoring BNPP hydrolysis catalyzed by Fe(III)−P$_1$</td>
<td>89</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>Comparison of saturation profiles of BNPP hydrolysis by several complexes of Fe(III) and synthesized polymers</td>
<td>91</td>
</tr>
<tr>
<td>Figure 3.10</td>
<td>Inhibition of BNPP hydrolysis catalyzed by Fe(III)−P1 by phosphate</td>
<td>94</td>
</tr>
<tr>
<td>Figure 3.11</td>
<td>Inhibition pattern for phosphate in BNPP hydrolysis catalyzed viii</td>
<td></td>
</tr>
</tbody>
</table>
by Fe(III)--P1

Figure 3.12  Oxidative DNA cleavage catalyzed by Fe(III)--P1

Figure 3.13  Structures of resins used in heterogeneous hydrolysis of BNPP

Figure 3.14  Initial rate of BNPP hydrolysis by Fe(III)--Chelex

Figure 3.15  Saturation profile of BNPP hydrolysis catalyzed by Fe(III)--Chelex and dependence of hydrolytic rate on [Fe(III)--Chelex]

Figure 3.16  Activity pH profile for BNPP hydrolysis by Fe(III)--Chelex

Figure 4.1  Structure based catalytic mechanism of tyrosinase

Figure 4.2  Three dimensional structure of *Ibomoea batatas* Catechol Oxidase

Figure 4.3  Proposed catalytic mechanism of cresolase and catecholase activity of Tyrosinase and Catechol Oxidase

Figure 4.4  Active centers of tyrosinase and structurally related proteins

Figure 4.5  Copper--dioxygen binding modes

Figure 4.6  Crystal structure active site of Hemocyanin and Kitajima’s complex

Figure 4.7  Three possible reaction mechanisms in phenol hydroxylation

Figure 4.8  Structures of flavonoids Quercetin, Catechin, Ellagic and Gallic acid

Figure 4.9  Structure of substrates containing a phenol moiety used in oxidative reactions

Figure 4.10  Structure of substrates containing a catechol moiety used in oxidative reactions

Figure 4.11  Formation of o--quinone and MBTH adduct

Figure 4.12  UV--vis spectra of reaction during oxidation of THB and phenol hydroxylation catalyzed by Cu(II)--P1

Figure 4.13  Rapid equilibrium binding scheme for random bisubstrate mechanism
| Figure 4.47 | Kinetic Isotope Effect in phenol hydroxylation by Cu(II)–P1 in the presence of O₂ and H₂O₂ | 219 |
| Figure 4.48 | Mechanistic Job Plot in Phenol hydroxylation by Cu(II)–P1 in presence of O₂ and H₂O₂ | 221 |
| Figure 4.49 | Effect of Sodium Borate on hydroxylation of Phenol and oxidation of Catechol by Cu(II)–P1 in air | 223 |
| Figure 4.50 | Effect of pH on hydroxylation of Phenol by Cu(II)–P1 in air and in H₂O₂ | 224 |
| Figure 4.51 | Binding of Phenol to the active site of Cu(II)–P1 using MM3 calculations | 226 |
| Figure 4.52 | Proposed Mechanism of Phenol hydroxylation in air and in H₂O₂ | 228 |
| Figure 4.53 | EPR spectra of Cu(II)–P1 in presence of various amounts of Zn(II) | 230 |
| Figure 4.54 | Comparison of lower field EPR spectra of Cu(II)–P1 in presence of various amounts of Zn(II) | 231 |
| Figure 4.55 | Inhibition of Dopamine oxidation by Quercetin Gallic and Ellagic acid | 233 |
| Figure 4.56 | Spectral changes during oxidation of various chlorinated phenols catalyzed by Cu(II)–P1 and comparison to self oxidation | 235 |
| Figure 4.57 | Changes in absorption during oxidation of 4–Chlorophenol catalyzed by Cu(II)–P1 and comparison to self oxidation | 238 |
| Figure 4.58 | Changes in absorption during oxidation of 2, 4–Dichlorophenol catalyzed by Cu(II)–P1 and comparison to self oxidation | 239 |
| Figure 4.59 | Changes in absorption during oxidation of 2, 4, 6–Trichlorophenol catalyzed by Cu(II)–P1 and comparison to self oxidation | 240 |
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>Am</td>
</tr>
<tr>
<td>Acrylic acid</td>
<td>Aa</td>
</tr>
<tr>
<td>2,2-azobisisobutyronitrile</td>
<td>AIBN</td>
</tr>
<tr>
<td>Carbon Nuclear Magnetic Resonance</td>
<td>$^{13}$CNMR</td>
</tr>
<tr>
<td>Correlation Spectroscopy</td>
<td>COSY</td>
</tr>
<tr>
<td>Heteronuclear Multiple Quantum Coherence</td>
<td>HMQC</td>
</tr>
<tr>
<td>Di-t-butyl catechol</td>
<td>DTC</td>
</tr>
<tr>
<td>Ethylene diamine tetra-acetate</td>
<td>EDTA</td>
</tr>
<tr>
<td>Electron Paramagneric Resonance spectroscopy</td>
<td>EPR</td>
</tr>
<tr>
<td>Molar absorptivity</td>
<td>$\varepsilon_o$</td>
</tr>
<tr>
<td>Methyl methacrylate</td>
<td>Mma</td>
</tr>
<tr>
<td>Number Average Molecular Weight</td>
<td>$M_n$</td>
</tr>
<tr>
<td>Proton Nuclear Magnetic Resonance Spectroscopy</td>
<td>$^{1}$HNMR</td>
</tr>
<tr>
<td>Poly (4-vinylpyridine–co–acrylamide)</td>
<td>$P_1$</td>
</tr>
<tr>
<td>Polydispersity</td>
<td>$M_w/M_n$, PD</td>
</tr>
<tr>
<td>Poly (methyl methacrylate)</td>
<td>PMMA</td>
</tr>
<tr>
<td>Trihydroxybenzene</td>
<td>THB</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>UV</td>
</tr>
<tr>
<td>Ultraviolet Visible Spectroscopy</td>
<td>UV-Vis</td>
</tr>
<tr>
<td>Vinylpyridine</td>
<td>Vp</td>
</tr>
</tbody>
</table>
Copper And Iron Complexes of Linear and Crosslinked Polymers as Catalysts For Phosphoester Hydrolysis and Oxidative Transformations of Catecholic and Phenolic Substrates

Vasiliki Lykourinou

**ABSTRACT**

The goal of this study is to utilize polymers as macromolecular ligands for the construction of catalysts by formation of coordination complexes with transition metals with the main focus on complexes of Cu(II) and Fe(III) and further determine (a) their catalytic efficiency (b) mechanism of action (c) similarities to enzymatic systems and synthetic metal complexes.

The reactions of interest are (1) hydrolytic cleavage of phosphoesters (2) oxidation of catechol type of substrates (3) hydroxylation of phenolic substrates and chlorinated phenols (4) activation of molecular oxygen and/ or hydrogen peroxide (5) oxidative cleavage of DNA plasmid.

The major premise of the study is that by mimicking the macromolecular nature and some structural features of enzymes, polymers can in principle, catalyze chemical transformations with similar efficiencies and specificities and can offer alternatives to
peptide based catalysts or simple metal complexes with the advantage of a wider range of building blocks, increased stability and the potential of reusability.

The crosslinked resins used contained the functional groups iminodiacetate (chelex resin), diethylenetriamine and tris(2-aminomethylamine) and were based on styrene–divinylbenzene backbone. The catalytic proficiencies of the Fe(III) and the Cu(II) complexes of chelex resin and diethylenetriamine approached 100 and 1000 respectively towards the model phosphodiester BNPP at pH 8.0 and 25 °C.

Moreover, the Fe(III) complexes of linear copolymers with repeating unit of three vinylpyridines to one acrylamide (P1) showed selectivity towards phosphodiester hydrolysis over monoesters and phosphonate esters and exhibited catalytic proficiencies approaching 50,000 towards BNPP hydrolysis. Further exploration of the catalytic capabilities of copolymer P1 revealed that Cu(II) complexes of this macromolecular ligand are potentially capable of assembling to active dicopper intermediates found in the catalytic pathways of copper oxygenases like tyrosinase and catechol oxidase and thus were able to accelerate catechol oxidation to ortho–quinones with rate accelerations approaching 10,000 and hydroxylate phenols with rate accelerations close to one million. The results suggest that these Cu(II)–polymer systems can potentially be used as model systems to further understand metal centered reactive oxygen species (ROS) generated in vivo and can be very promising remediation agents for the dechlorination of persistant chlorine containing pollutants.
CHAPTER 1. INTRODUCTION

I. Metallopolymers

Polymers are ubiquitous in the manufacture of a large number of materials commonly used in the modern society such as plastics, fibers, elastomers, adhesives, rubber and prosthetics to name a few. Furthermore, many biologically important molecules such as nucleic acids, proteins and carbohydrates are also polymeric in nature making macromolecular structures one of the most commonly occurring. Various ways of synthesis and fabrication of polymeric substances have been established, allowing the synthesis of a wide variety of products used in a range of applications.

The realization that incorporation of metals in polymeric substances can vary the properties of the resulting material opened the field to new possibilities. Research on metal-containing polymers began in the early 1960's after it was established that vinyl ferrocene and other vinylic transition metal complexes can undergo polymerization under the same conditions as organic monomers (Fig. 1) leading to the formation of polymers incorporating a potentially reactive metal in the polymeric structure. ¹

![Figure 1. Polymerization of vinyl ferrocene](image-url)
Some of these metal containing polymeric materials could act as semi-conductors and possessed one or two dimensional conductivity (Figure 2) making their applications in electronics possible.\textsuperscript{2} It was also realized that reactions used to make simple metal chelates could be used to prepare polymers if the ligands were designed properly, such as incorporation of vinyl groups in the ligand structure.\textsuperscript{3} Furthermore, the incorporation of transition metals into the polymer main chain offers a unique potential for the preparation of materials that can be further processed much like conventional polymers, an advantage not available in simple metal complexes, offering material, with new properties which differ significantly from those of conventional carbon-based polymers. For example, the diverse range of coordination numbers and geometries available for transition elements offer the possibility of accessing polymers with unusual conformational, mechanical, and morphological characteristics.\textsuperscript{4}

Apart from their use for the synthesis of new materials with enhanced properties, it was realized that metal containing polymers are well suited for the design of heterogeneous catalysts that can potentially be recovered and reused due to their macromolecular nature. As interest in homogeneous catalysts developed in the late 60's and early 70's, several investigators began binding homogeneous catalysts onto polymers,\textsuperscript{5} where the advantage of homogeneous catalysis – with known reaction mechanisms and the advantage of heterogeneous catalysis – such as simplicity and ease of recovery of catalysts could both be achieved. Indeed, the polymer matrix itself often enhanced the selectivity of the catalyst.\textsuperscript{6} Over the years, there have been several approaches for the design and synthesis of novel metal- polymer catalysts.
Figure 2. Mechanism of electron transfer in conducting polymer systems
(adopted from ref. 2)
II. Classification of metal complexes and metals in macromolecules

Metals or metal complexes can be a part of the macromolecular chain or network in more than one ways. The metal can form a bond with a macromolecule, it can be a part of it via the ligand or it can be physically incorporated into the macromolecule. These interactions can be classified to three main categories.

- Type I. A metal ion is bound to a chain of a linear or cross-linked macromolecule via a covalent, coordination, ionic, or π-bond (Fig. 3).

![Diagram of metal complexes and bonds](image)

**Figure 3.** Metals bound at macromolecules
In this type of interaction, the secondary forces of the bound metal to other parts of the chain are weak (hydrophobic interactions, hydrogen bonds, charge interactions) but because they are multiple, they play a cooperative role. Furthermore, they are affected by the conformational changes of the macromolecular environment. Some examples of type I interactions include binding of metal to rigid cross linked organic polymer, or at the internal surface of porous materials such as zeolites or silica. Some of the applications of such materials includes gas transport, sensing, ion-exchange resins, catalysis, photocatalysis, and biosensors.

- **Type 2:** The ligand of a metal complex is part of a macromolecular chain or network. The preparation of this type of metallopolymer can be achieved by polymerization of low molecular weight metal complexes precursors or the macromolecular ligand can be prepared first and then metallated in the second step. Examples of this type are shown in Fig. 4.

![Figure 4. Ligand of a metal complex as a part of a macromolecule.](image-url)
• **Type 3:** In this case the metal or a metal complex is part of a macromolecular chain or network made strictly from metal atoms. Schematically, this type is the direct opposite of the one depicted in Fig. 4. The metal is part of a macromolecular chain or network.

In this type of interactions, the metal or the ligand are part of a rigid macromolecular network, therefore they have a fixed coordination and conformational changes are restricted. This material is well suited for integrated electronic processes. Typical uses of these material includes conductors and photoconductors, photovoltaic cells, light emitting diodes, electrochemical cells.

• **Type 4.** In this category, metal clusters or metal complexes are physically incorporated into macromolecules. The macromolecular environment can potentially stabilize the metal clusters and leads to the generation of new composite materials (Fig. 5).

![Figure 5. Physical incorporation of metal nano-particles (left) or metal complexes (right)](image-url)
The properties of these materials are greatly affected by the macromolecular environment. Typical uses of these materials include magnetic nanocomposites and metallized plastics, among a few. In the case where metal complexes are incorporated into the macromolecular structure, the resulting material is used in thin film devices for conductors and photoelectrocatalysts.

### III. Metal containing biomacromolecules

A great variety of metals are used in biological systems ranging from alkali to transition metals and are essential in various processes of growth and metabolism. Metal ions interact with a variety of biopolymers through ionic, coordination and covalent bonds. Proteins provide several sites for metal interaction such as the (C=O) group and N, O, S donor atoms from the side chains. Metal complexes of biomacromolecules play an important role in various processes essential in life sustenance such as oxygen transport by hemoglobin and myoglobin, energy conversion through photosynthesis, electron transfer through cofactors, and catalysis through the action of various metalloenzymes. Moreover, some metal ions are essential at low levels but can be toxic at elevated concentrations while some metals are toxic at all concentrations due to their effect on biological molecules such as enzymes by causing alterations in their normal function. Understanding these metal interactions is very important not only in explaining their role in healthy functional cells but also in deciphering their ability to trigger disease. Moreover, due to the importance of metals in the optimal function of metalloenzymes, nature’s catalysts, efficient synthetic catalysts can be designed by mimicking the coordination environment provided by the protein backbone to biologically essential metals.
IV. Polymer based heterogeneous catalysis

Heterogeneous catalysts are frequently defined as solids or mixtures of solids capable of accelerating chemical reactions without undergoing changes. Catalysts and reagents immobilized on a range of insoluble supports have been utilized and reported since the late 1960’s. The main benefits are due to the ease of physical separation of the polymer and its bound component from the reaction mixture, the ease of recycling (especially with expensive catalysts and ligands) and the simplification of handling a range of toxic materials. Such immobilization also enable the use of high concentrations of catalysts to drive reactions to completion, as excess reagents can be removed by filtration, often eradicating the need of time-consuming purification steps. Another advantage is due to the unique microenvironment created for the reactants within the polymer support. Improved catalyst stability within the polymer matrix,\textsuperscript{9} increased selectivity for intramolecular reactions,\textsuperscript{10} increased regioselectivity due to steric hindrance,\textsuperscript{11} and the superior activity of some supported chiral catalysts due to site cooperation are some of the advantages offered by macromolecular catalysts.

A very promising approach to the design of heterogeneous catalytic materials has been the translation of the main principles of enzyme catalysis in an effort to design novel catalytic materials. The advantages of these ‘artificial enzymes’ can be substrate specific and reaction–selective much like their natural counterparts but with the added benefit of better accessibility, stability, and a broader variety of catalyzed reactions.

Of special interest in enzyme mimics would be the use of synthetic polymeric substances, since these compounds are usually very stable against heat, chemicals and solvents and they can easily be fabricated in a form suitable for industrial applications.
The use of polymers makes the system more complicated compared to its low molecular weight counterparts since the support needs to be prepared in a defined three-dimensional structure. On the other hand, polymers offer certain advantages if the macromolecular nature of enzymes is taken into consideration. In fact, many of the unique features of enzymes are directly related to their polymeric nature. This is particularly true for the high cooperativity of the functional groups and the dynamic effects such as the induced fit, the allosteric effect and the steric strain exhibited by enzymes.

Several approaches have been utilized to introduce catalytically active sites into polymers mostly by copolymerization of the appropriate monomers (Fig. 6) bearing the desired catalytic functionalities (e.g. imidazole, OH and COOH). This method provides a polymer with randomly distributed functional groups (Fig. 6A). Another possibility involves the attachment of side chains, containing the desired arrangement of functional groups onto the parent polymer (Fig 6B) or a third possibility is the polymerization or polycondensation of monomers with the desired linear arrangement of functional groups. In this case, the groups are localized in the main chain one after another, as in certain hormone receptors (Fig. 6C). The first two approaches led to limited success due to the difficulty in orienting the functional groups needed for optimum catalysis in the proper orientation. The most promising approach to enzyme mimics requires the use of scheme Fig.6D as it is the closest to a typical arrangement in a protein active site.
Figure 6. Possible arrangements of functional Groups in synthetic and natural polymers (adopted from ref. 12).
V. Linear and crosslinked copolymers and their metal complexes in catalysis

A great variety of macromolecules can be employed for binding of metals and construction of potential catalytic sites. Two main approaches can be used to obtain metallopolymers for catalytic applications.

- Linear organic polymers with the appropriate functional groups can be used as ligands for chelation of appropriate metal ions. For their preparation, the monomers for the synthesis of the polymer-ligand can be chosen so as to obtain polymers that are soluble in most common solvents. The use of such a macromolecular ligand can in principle behave much like the polypeptide chains of proteins assembling into an active enzyme by orienting around the suitable metal ion necessary for its proper function. This approach is an extension of the well established use of small transition metal complexes to model the active site of metalloenzymes commonly used by bioinorganic chemists in their effort to understand the properties of the metal relevant to the catalyzed reaction.

- Cross-linked insoluble organic copolymers can be prepared by polymerization of a monovinyl compound functionalized with groups for interaction with the metal and a divinyl comonomer for cross-linking (often 1,2-divinylbenzene or 1,2-ethanedioldimethacrylate). A high degree of crosslinking allows resins capable of swelling while a low degree of crosslinking affords macroporous resins with a permanent pore structure.

Both approaches can potentially lead to the formation of metallo–macromolecular catalysts that can be heterogeneous due to their insolubility in the reaction medium.
However, it is worth noting that linear copolymers offer the advantage of flexibility in assembling of potential reactive intermediates which may not be always feasible in the case of crosslinked resins.

VI. Advantages and disadvantages in use of polymeric catalysts.

Functionalized polymer materials in general have received much attention and some of their most noted applications include Merrifield’s solid-phase synthesis\(^\text{13}\) of peptides, their use as polymer-immobilized catalysts,\(^\text{14}\) immobilization of enzymes,\(^\text{15}\) and ligand exchange chromatography. A recently reported application suggests the potential for selective protein recognition by synthetic receptor macromolecules based on copolymers of simple binding monomers.\(^\text{16}\) This approach, inspired by protein-protein interactions, makes use of the fact that large contact areas where polar and hydrophobic interactions can be maximized are very important in protein interactions and macromolecular hosts can mimic these interactions very effectively opening the possibility for new applications of polymers.

Some of the main advantages of polymeric catalysts include the following:

- Immobilization of the catalytic center usually increases efficiency because their concentration on the polymer support is not limited by solubility.
- Use of metallopolymer catalysts allows the use of higher temperatures.
- The ‘local concentration’ of active center is high.
- The catalyst can be recycled leading to a more cost effective system.
Some of the disadvantages of the polymeric systems are:

- Polymeric materials typically consist of a distribution of chain lengths and molecular weights leading to an inhomogeneity or ‘polyclonality’ of the catalytic sites.
- While small molecules tend to be crystalline due to the ability of the molecules to pack themselves in regular three-dimensional arrays, polymers can be amorphous, totally lacking positional order on the molecular scale.
- Lack of order on the molecular scale can make the characterization of the active sites very difficult since crystal structures of the active catalysts cannot be obtained.

Despite the difficulties in characterization of polymeric catalysts, new developments in polymer science provides ways to overcome their limitations. In this dissertation, the use of polymer based catalysts for hydrolytic reactions is explored using crosslinked polymeric material as support for catalytic metal centers. Furthermore, the emphasis is placed on linear copolymers of vinylpyridine as the primary ligand for metal binding polymerized with a variety of monomers chosen as potential substrate recognition sites due to their hydrophobic nature (styrene, methylmethacrylate), the existence of a charged group in their structure (acrylic acid) or due to their hydrogen bonding capabilities (acrylamide).
References


CHAPTER 2. SYNTHESIS AND METAL BINDING OF LINEAR COPOLYMERS
(VINYLPYRIDINE-co-X)\textsubscript{n} PREPARED VIA FREE RADICAL POLYMERIZATION
(X = ACRYLAMIDE, METHYL METHACRYLATE, STYRENE, ACRYLIC ACID)

I. Introduction

1. Free radical polymerization

Polymers are ubiquitous in the construction of numerous materials used in modern society and it is appropriate to state that the advances in science and technology of materials are parallel with advances in polymer science. Free radical polymerization is the most widespread method of polymerization of vinylic monomers and accounts for more than 50% of plastics produced in the world.\footnote{1} The carbon-carbon double bond, due to its low stability, is very susceptible to attack by a free radical. The type of compounds represented by the general formula CH\textsubscript{2}=CHX and CH\textsubscript{2}=CXY are called vinyl monomers and react well with free radicals.

Not all vinyl monomers yield polymers via radical polymerization. Aliphatic hydrocarbons other than ethylene polymerize to oils and 1,2-disubstituted ethylenes do not polymerize at all.\footnote{2} Free radical polymerizations are chain reactions in which every polymer chain grows by addition of a monomer to the terminal free radical reactive site called “active center”. The addition of the monomer to this site induces the transfer of the active center to the newly created chain end. The mechanism of the free radical
polymerization can be divided into three distinct stages referred to as a) initiation b) propagation and c) termination.

a) Initiation: The initiation step is considered to involve two reactions creating the free radical active center. The first event is the production of free radicals. This can be done by several mechanisms such as thermal, redox or photochemical reactions. The most common case is the homolytic dissociation of an initiator I to yield two radicals R• often referred as primary radical. The subsequent addition of this radical to a monomer molecule M produces the initial propagating species RM•. Since the formation of R• proceeds more slowly than the reaction of R• with monomer, the first step is rate-determining.

b) Propagation: Propagation takes place by the successive addition of a large number of monomer molecules M to the primary radical RMn•. Each rapid sequential addition of a monomer molecule creates a new radical RM_{n+1}• that is larger by one monomer unit. The propagation usually takes place rapidly.

c) Termination: In this step, the growth of the polymer chain is terminated irreversibly. The two most common mechanisms for termination involve bimolecular reactions. The first one, combination, is the reaction between two growing polymer chains leading to the annihilation of the radical active centers and the formation of a single polymer chain. The second one is the termination via disproportionation. A hydrogen atom is abstracted from a growing chain and transferred to another one. This reaction leads to two terminated polymer chains, one with an unsaturated end-group and the other one with a saturated end-group.³
**Initiation**

Initiator → 2R●

R● + Monomer (M) → RM●

Rate = \( k_1[M][R●] \)

**Propagation**

RM● + M → RMM●

Rate = \( k_p [RM●][M] \)

**Termination**

Polₐ● + Polₐ● → Polymer

Rate = \( k_{tc}[Polₐ●]^2 + k_{td}[Polₐ●]^2 \)

R● + R-CH₂-CH₂● → RH + R-CH=CH₂

**Figure 2.1.** Individual steps in free radical polymerization
Various types of initiators have been used in free radical polymerization. Some monomers such as styrene and methylethacrylate and some strained cycloalkenes can undergo polymerization without the addition of free radical initiators. Most other monomers require initiator for the polymerization to take place. The four major types of initiators for free radical polymerizations are:\(^4\)

(a) peroxides and hydroperoxides

(b) azo compounds

(c) redox initiators and

(d) photoinitiators.

A common azo initiator used in free radical polymerizations is 2,2-azobisisoburyronitrile (AIBN) (Figure 2.2). Some of the advantages of azo initiators are their half lives of \(~10\) hours from 44 °C to 111 °C,\(^5\) easily controlled polymerization reactions, synthesis of high molecular weight with high linearity. Furthermore, they are minimally affected by other components in the reaction mixture, allow for end group functionalization, and are easy to store and handle. Some of the factors that affect initiator efficiencies are

- Reaction with oxygen, solvent, impurities
- Primary-primary radical combination
- Decomposition

The relationship of reaction temperature and initiator efficiency for AIBN can be calculated using the dissociation constant \(k_d = 6.02096 \times 10^{-5} \text{ s}^{-1}\) of AIBN at 71.2°C by the following formulas:
\[
t_{1/2} = \frac{0.693}{k_d} \quad \text{Eq. 1}
\]

\[
\log k_{d2} = \log k_{d1} - \left[ \frac{E_a(T_2-T_1)}{2.303RT_2T_1} \right] \quad \text{Eq. 2}
\]

where \(T_1\) and \(T_2\) are two different reaction temperatures, \(R\) is the gas constant and \(E_a\) is the activation energy.

**Figure 2.2.** Decomposition of 2, 2,-azobis(isobutyronitrile) (AIBN) to the dimethyleyano free radical.
<table>
<thead>
<tr>
<th>Half life (t_{1/2})</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 hours</td>
<td>64</td>
</tr>
<tr>
<td>1 hour</td>
<td>82</td>
</tr>
<tr>
<td>6 min</td>
<td>100</td>
</tr>
<tr>
<td>1 min</td>
<td>120</td>
</tr>
</tbody>
</table>

**Table 2.1.** Half-life of AIBN as a function of reaction temperature

The amount of initiator can determine the molecular weight of the resulting polymer. The amount of radicals present, in particular, is the determining factor. If the radicals generated are too few, poor conversion occurs whereas if too many are generated the reaction is exothermic and the resulting molecular weight is low. This is related to the *average kinetic chain length*, \( V \), which is defined as the average number of monomer units polymerized per chain initiated. The average kinetic chain length is given by the eq. 3, where \( k_p \) is the rate constant for propagation, \( k_t \) the rate constant for termination and \( k_d \) the rate constant for initiator decomposition, \([I]\) is the initiator concentration and \([M]\) is the monomer concentration.

\[
V = \frac{k_p[M]}{2(f k_t k_d[I])^{1/2}} \quad \text{Eq. 3}
\]

Thus, varying initiator concentration provides one way of controlling molecular weights of polymers.
Figure 2.3. Polymer structural elements (Adopted from reference 16)
2. Copolymerization

The mechanism of copolymerization is analogous to that of homopolymerization, however, the reactivities of the participating monomers can vary significantly. In this type of reactions, the monomers can undergo either self-propagation or cross-propagation and the extent to which the monomers participate in each of these reactions will ultimately determine the composition of the resulting copolymers. The quantities \( r_1 \) and \( r_2 \) are the *reactivity ratios* defining the relative tendencies of the monomers to self-propagate or cross-propagate with \( r_1 > 1 \) when \( M_1 \) tends to self-propagate and \( r_1 < 1 \) when copolymerization is preferred. If \( f_1 \) and \( f_2 \) are the mole fractions of monomers \( M_1 \) and \( M_2 \) and \( F_1 \) and \( F_2 \) are their mole fractions in the copolymer, there exist a mathematical relationship among all these parameters shown in eq.5.

\[
F_1 = r_1 f_1^2 + f_1 f_2 / r_1 f_1^2 + 2f_1 f_2 + r_2 f_2^2 \quad \text{Eq. 5}
\]

\[
F_2 = 1 - F_1
\]

The knowledge of reactivity ratios is very important in the determination of feed ratios in copolymer preparations. A semi-empirical relationship has been developed in order to make the determination of reactivity ratios simpler. This relationship, known as *Q-e scheme*, can relate the reactivity ratios with constants independent of the comonomer and solely dependent on the characteristics of each individual monomer shown in eq. 6 and 7.
where $Q$ is a measure of the reactivity of each monomer and are related to resonance stabilization of monomer and $e$ is a measure of the polarity of the monomers. Styrene is the standard and is assigned values of $Q = 1.00$ and $e = -0.8$. The values of $Q$ increase with increasing resonance stabilization and the $e$ values become less negative as the groups attached to the double bond become more electron attracting. 

The values of $Q$ and $e$ for the monomers used in this study are given in Table 2.2.
Table 2.2 Values of Q and e parameters for monomers used in copolymer synthesis

<table>
<thead>
<tr>
<th>Monomer</th>
<th>Q</th>
<th>e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (Am)</td>
<td>1.120</td>
<td>1.190</td>
</tr>
<tr>
<td>Acrylic acid (Aa)</td>
<td>1.150</td>
<td>0.770</td>
</tr>
<tr>
<td>2-vinylpyridine (2Vp)</td>
<td>1.300</td>
<td>−0.500</td>
</tr>
<tr>
<td>4-vinylpyridine (4Vp)</td>
<td>1.000</td>
<td>−0.280</td>
</tr>
<tr>
<td>Methylmethacrylate (Mma)</td>
<td>0.740</td>
<td>0.400</td>
</tr>
<tr>
<td>Styrene (Sty)</td>
<td>1.000</td>
<td>−0.800</td>
</tr>
</tbody>
</table>

Using the parameters mentioned above it is possible to estimate the repeating unit
of the resulting polymer for a given feed ratio and compare it to the synthesized polymers.

<table>
<thead>
<tr>
<th>Monomer feed ratio</th>
<th>Calculated Repeating Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>25:75 %</td>
<td>( F_1:F_2 )</td>
</tr>
<tr>
<td>4VP:AM</td>
<td>1 : 1.3</td>
</tr>
<tr>
<td>4VP:AA</td>
<td>1 : 2</td>
</tr>
<tr>
<td>4VP:MMA</td>
<td>1 : 2</td>
</tr>
<tr>
<td>4VP:STY</td>
<td>1 : 2</td>
</tr>
</tbody>
</table>

*Table 2.3.* Calculated repeating units of the synthesized polymers
II. Experimental

1. Monomers and initiator used in polymerization

A series of copolymers was synthesized using different molar ratios of monomers (shown in Fig. 2.4) and initiator AIBN. A typical polymerization reaction contained 2- or 4-vinylpyridine and one other vinylic monomer shown in Fig. 2.4. The molar ratios of each monomer couple used (also known as ‘feed ratio’) was 25:75, 50:50 and 75:25 respectively. The initiator used was calculated based on the total moles of monomers for each reaction and it was varied while the monomer ratio was kept constant in order to produce polymers of different size containing the same repeating units.

Figure 2.4. Structure of monomers used in synthesis of copolymers.
<table>
<thead>
<tr>
<th>Reactant</th>
<th>Density</th>
<th>mp(°C)</th>
<th>bp(°C)</th>
<th>FM (grams/mole)</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide¹ (AM)</td>
<td>1.051</td>
<td>84-86</td>
<td>125/25</td>
<td>71.08</td>
<td>Benzene, chloroform</td>
</tr>
<tr>
<td>Acrylic acid² (AA)</td>
<td>0.975</td>
<td>13</td>
<td>139</td>
<td>72.06</td>
<td></td>
</tr>
<tr>
<td>4-Vinylpyridine³ (4VP)</td>
<td>0.975</td>
<td>62-65</td>
<td>/15mmHg</td>
<td>105.14</td>
<td>DMF, DMSO</td>
</tr>
<tr>
<td>2-Vinylpyridine⁴ (2VP)</td>
<td>0.975</td>
<td>79-82</td>
<td>/29mmHg</td>
<td>105.14</td>
<td>DMF, DMSO</td>
</tr>
<tr>
<td>Styrene</td>
<td>0.905</td>
<td></td>
<td></td>
<td></td>
<td>Sparingly soluble in water, MeOH, ether, alcohols, acetone</td>
</tr>
<tr>
<td>Methyl Methacrylate (MMA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>THF, esters, methylethylketon, aromatic and chlorinated H/C</td>
</tr>
<tr>
<td>2,2’- Azobisisobutynitrile (AIBN)</td>
<td>103-105</td>
<td>164.2</td>
<td></td>
<td>164.2</td>
<td>Methanol, DMSO</td>
</tr>
</tbody>
</table>

*Table 2.4. Monomers used in free radical polymerization and their properties*
2. Copolymer synthesis

a. Vinylpyridine-co-acrylamide series

The molar ratios of initiator to total moles monomer used were 1:1000, 1:100, 1:50 and 1:20. The monomers and initiator were dissolved in minimum amount of DMF (2 mL were used for total weight of ~1.2 g). The reaction can be performed in larger scale but it is not recommended that the total weight of all the reactants exceed 10.0 grams because the reaction is exothermic and cannot be controlled if this amount is exceeded.

- Nitrogen is bubbled in the reaction mixture for 10 min using pasteur pipette adaptor in an unsealed tube, followed by 15 min sonication and another 10 min of nitrogen bubbling in sealed tube to avoid oxygen.

- The reaction mixture is heated in oil bath at 90-100°C for 1 hour. The sample containing 1:1000 initiator was heated for 3 hours. The samples containing higher amounts of initiator were heated to the lowest temperature. In the case of copolymers prepared using 1:20 (when 1:20 initiator is used the reaction is complete after heating for 15 min at 70°C). The samples are generally heated until the reaction mixture becomes very viscous.

- The polymer is dissolved in minimum amount of methanol and precipitated in ethylacetate. The polymer solution is added dropwise in ethylacetate under vigorous stirring. To ensure removal of unreacted monomers this procedure must be repeated twice. The purified polymer is dried in vacuum oven at 40°C.

The 2-vinylpyridine–acrylamide series is prepared under the same conditions as the 4-vinylpyridine–acrylamide. A series of copolymers containing 4-vinylpyridine and
acrylamide in molar ratio of 25:75 respectively were prepared. In order to vary the size of the resulting polymer, four molar ratios of initiator were used while keeping the molar ratio of monomers constant generating four polymers. The amounts of each reactant used are given in Table 2.6.

4VP-co-AM (25:75)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Initial mole ratio</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP/2VP</td>
<td>0.25</td>
<td>1.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.98 mL)</td>
</tr>
<tr>
<td>AM</td>
<td>0.75</td>
<td>2.03</td>
</tr>
<tr>
<td>Initiator</td>
<td>1:1000</td>
<td>0.00625</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.0625</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>0.313</td>
</tr>
<tr>
<td>Total Wt (g)</td>
<td>(1:1000)</td>
<td>3.04</td>
</tr>
<tr>
<td></td>
<td>(1:100)</td>
<td>3.10</td>
</tr>
<tr>
<td></td>
<td>(1:50)</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td>(1:20)</td>
<td>3.35</td>
</tr>
</tbody>
</table>

Table 2.5. Mole ratios and monomer and initiator weights used in synthesis of the 4 Vp-co-Am 25:75 copolymer series.
The same procedure was repeated using a 50:50 molar feed ratio of 4-vinylpyridine to acrylamide and varying the amount of initiator as above. The amounts of each reactant used in the four reactions are given in Tables 2.7.

### 4VP-co-AM (50:50)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Initial mole Ratio</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP/2VP</td>
<td>0.50</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.72 mL)</td>
</tr>
<tr>
<td>AM</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Initiator</td>
<td>1:1000</td>
<td>0.00231</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.0231</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.0462</td>
</tr>
<tr>
<td>Total Wt (g)</td>
<td>(1:1000)</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>(1:100)</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>(1:50)</td>
<td>1.29</td>
</tr>
</tbody>
</table>

**Table 2.6.** Mole ratios and total weight of reactants used in the synthesis of the initiators weights used in synthesis of the 4VP-co-Am 50:50 copolymer series
The third series was synthesized using a 75:25 molar feed ratio of 4-vinylpyridine to acrylamide and varying the amount of initiator as above. The amounts of each reactant used in the four reactions are given in Tables 2.8.

### 4VP-co-AM (75:25)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Initial Mole Ratio</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP/2VP</td>
<td>0.75</td>
<td>2.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.06 mL)</td>
</tr>
<tr>
<td>AM</td>
<td>0.25</td>
<td>0.46</td>
</tr>
<tr>
<td>Initiator</td>
<td>1:1000</td>
<td>0.0042</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.084</td>
</tr>
<tr>
<td>Total Wt (g)</td>
<td>(1:1000)</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
<td>(1:100)</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>(1:50)</td>
<td>2.56</td>
</tr>
</tbody>
</table>

**Table 2.7.** Mole ratios and monomer and initiator weights used in synthesis of the 4Vp-co-Am 75:25% copolymer series
b. *vinylpyridine-co-acrylic acid series*

The acrylic acid co-polymers are prepared without heating. This reaction is very exothermic and the reaction tubes need to be kept in water-ice bath to avoid complete decomposition of the product. Polymer formation begins after oxygen is driven out of the sealed tube by bubbling nitrogen for 5 minutes. Tables 2.9 through 2.11 show the series synthesized using a 75:25 molar feed ratio of 4-vinylpyridine to acrylic acid and varying the amount of initiator as above. The amounts or each reactant used in the four reactions are given in Tables 2.8.

The polymer is dissolved in methanol and precipitated in ethylacetate.

**4VP-co-AA (25:75)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Initial Mole Ratio</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP</td>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>(0.98 mL)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.75</td>
<td>2.0</td>
</tr>
<tr>
<td>Initiator</td>
<td>1:1000</td>
<td>0.0062</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.125</td>
</tr>
<tr>
<td>Total Wt</td>
<td>(1:1000)</td>
<td>3.01</td>
</tr>
<tr>
<td>(g)</td>
<td>(1:100)</td>
<td>3.06</td>
</tr>
<tr>
<td></td>
<td>(1:50)</td>
<td>3.13</td>
</tr>
</tbody>
</table>

**Table 2.8.** Mole ratios and monomer and initiator weights used in synthesis of the 4Vp-co-Aa 25:75% copolymer series
### 4VP-co-AA (50:50)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mole Ratio</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP</td>
<td>0.50</td>
<td>2.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.83 ml)</td>
</tr>
<tr>
<td>AA</td>
<td>0.50</td>
<td>2.0</td>
</tr>
<tr>
<td>Initiator</td>
<td>1:1000</td>
<td>0.0091</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.182</td>
</tr>
<tr>
<td>Total Wt</td>
<td>(1:1000)</td>
<td>4.92</td>
</tr>
<tr>
<td>(g)</td>
<td>(1:100)</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>(1:50)</td>
<td>5.1</td>
</tr>
</tbody>
</table>

**Table 2.9.** Mole ratios and monomer and initiator weights used in synthesis of the 4VP-co-Aa 50:50% copolymer series
### 4VP-co-AA (75:25)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mole Ratio</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP</td>
<td>0.75</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.3ml)</td>
</tr>
<tr>
<td>AA</td>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td>Initiator</td>
<td>1:1000</td>
<td>0.0091</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>0.182</td>
</tr>
<tr>
<td>Total Wt</td>
<td>(1:1000)</td>
<td>5.41</td>
</tr>
<tr>
<td>(g)</td>
<td>(1:100)</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>(1:50)</td>
<td>5.6</td>
</tr>
</tbody>
</table>

**Table 2.10.** Mole ratios and monomer and initiator weights used in synthesis of the 4Vp-co-Aa 75:25% copolymer series
c. Preparation of vinylpyridine-co-styrene and vinylpyridine-co-
methylmethacrylate series

The vinylpyridine-co-styrene copolymer was synthesized using pyridine and styrene in a ratio of 25 : 75 with initiator AIBN of 1:100 mole ratio to the total moles of monomers. The polymer is prepared by heating at 75°C for 2 hours after degassing and nitrogen bubbling with same procedures as followed for the polymer series above. The product of polymerization is dissolved in toluene and precipitated in hexane. Likewise, 4-vinylpyridine and methylmethacrylate were mixed in ratio of 25:75 using 1:100 molar ratio of initiator and heating at at 60°C for 3 hours. The resulting copolymer is dissolved in acetone and precipitated in water. The amount of reactants used for the each of the reactions are given in Table 2.12.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Initial Mole Ratio</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP</td>
<td>0.25</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Styrene</td>
<td>0.75</td>
<td>2.37 g</td>
</tr>
<tr>
<td>MMA</td>
<td>1:100</td>
<td>3.02 mL</td>
</tr>
<tr>
<td>Initiator</td>
<td>1:100</td>
<td>0.0625</td>
</tr>
<tr>
<td>Total Wt (g)</td>
<td>(Styrene)</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>(MMA)</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Table 2.11. Mole ratios and monomer and initiator weights used in synthesis of the 4VP-co-Styrene and 4VP:MMA 25:75% copolymer series
d. Product yields

<table>
<thead>
<tr>
<th></th>
<th>4VP:AM</th>
<th>% YIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>25:75</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initiator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1000</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td>86.2 / 87.5</td>
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<td>1:50</td>
<td></td>
<td>33.5</td>
</tr>
<tr>
<td>1:20</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td><strong>50:50</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initiator</td>
<td></td>
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<td>1:1000</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>1:100</td>
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<td>76.2 / 52.0</td>
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<td>1:50</td>
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<td>85.2</td>
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<td><strong>75:25</strong></td>
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<tr>
<td>Initiator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1000</td>
<td></td>
<td>43.7</td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td>74.0 / 84.3</td>
</tr>
<tr>
<td>1:50</td>
<td></td>
<td>84.6</td>
</tr>
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</table>

**Table 2.12.** Product yields in the polymerization of 4Vp:Am copolymers
<table>
<thead>
<tr>
<th>Initiator</th>
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<tbody>
<tr>
<td>1:1000</td>
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<td></td>
</tr>
<tr>
<td>1:50</td>
<td>70.2</td>
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</tbody>
</table>

**50:50 2VP:AM**

<table>
<thead>
<tr>
<th>Initiator</th>
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</thead>
<tbody>
<tr>
<td>1:1000</td>
<td>29.0</td>
<td></td>
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<tr>
<td>1:100</td>
<td>56.5</td>
<td></td>
</tr>
<tr>
<td>1:50</td>
<td>60.0</td>
<td></td>
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</tbody>
</table>

**75:25 2VP:AM**

<table>
<thead>
<tr>
<th>Initiator</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1000</td>
<td>31.7</td>
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<tr>
<td>1:100</td>
<td>67.3</td>
<td></td>
</tr>
<tr>
<td>1:50</td>
<td>35.5</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.13.** Product yields in the polymerization of 2Vp:Am copolymers
<table>
<thead>
<tr>
<th>4VP:AA</th>
<th>25:75</th>
</tr>
</thead>
<tbody>
<tr>
<td>INITIATOR</td>
<td>% YIELD</td>
</tr>
<tr>
<td>1:1000</td>
<td>68.4</td>
</tr>
<tr>
<td>1:100</td>
<td>72.7</td>
</tr>
<tr>
<td>1:50</td>
<td>71.0</td>
</tr>
<tr>
<td>4VP:AA</td>
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<tr>
<td>1:1000</td>
<td>68.0</td>
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<td>1:100</td>
<td>74.0</td>
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<td>1:50</td>
<td>47.0</td>
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<td>4VP:AA</td>
<td>75:25</td>
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<td>1:1000</td>
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<tr>
<td>1:100</td>
<td>68.6</td>
</tr>
<tr>
<td>1:50</td>
<td>40.1</td>
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**Table 2.14.** Product yields in the polymerization of 4Vp:Aa copolymers
<table>
<thead>
<tr>
<th>4VP:STYRENE</th>
<th>INITIATOR</th>
<th>% YIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td>25:75</td>
<td>1:100</td>
<td>58.8</td>
</tr>
<tr>
<td>4VP:MMA</td>
<td>25:75</td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td>50.0</td>
</tr>
</tbody>
</table>

**Table 2.15.** Product yields in the polymerization of 4Vp:Mma and 4Vp:Styrene copolymers
III. Results and Discussion

A. Polymer characterization

1. Repeating unit determination using $^1$HNMR

Nuclei in solution relax when they lose energy by passing from the high energy $N_b$ to the low energy state $N_a$. Molecular tumbling rates of $10^8$ s$^{-1}$ cause the $^{13}$C and $^1$H nuclei to efficiently relax through dipolar relaxation. The effect of efficient relaxation on the appearance of the NMR spectrum is to broaden the lines. In small molecules with a molecular weight close to 100 Da, the tumbling frequency is too fast ($\sim 10^{-11}$ s$^{-1}$) to promote efficient relaxation and the NMR lines appear sharp. In larger molecules with molecular weights of about 1,000 Da or more the tumbling frequency is approximately $10^{-9}$ s$^{-1}$ or less and the lines broaden with increasing molecular weight. The formation of the polymers can thus be verified with $^1$H NMR spectra, which exhibit very broad signals of up to $\sim$500 Hz for the polymers and the disappearance of the sharp monomer signals.

All the synthesized polymers contained (4– or 2–) vinylpyridine in the polymer repeating units, which was chosen to confer optimal metal binding capabilities to the synthesized polymers. The 4–vinylpyridine moiety gives two signals in the 6-8 ppm region of the proton NMR spectra. The existence of two distinct chemical shifts attributed to the pyridine moiety allows the determination of the repeating unit of the synthesized copolymers. By integration of the vinylpyridine proton signals and comparison to the integration of protons present in functional groups of the other monomers used in the copolymer synthesis, it is possible to determine the molar ratio of the substituents in the polymer average repeating unit (RU).
The two protons in the ortho- position of the ring reference to the vinyl group appear more upfield (6.8 ppm) compared to the two protons in the meta- position (8.3 ppm) Fig. 2.5. The acrylamide signals appear at 6.4 and 6.9 ppm which overlap with the ortho-pyridine signals. For the vinylpyridine (Vp) and acrylamide (Ac) containing copolymers depending on the monomer feed ratio and the amount of the initiator in the preparation, copolymers with different amounts of Vp and Ac can be obtained. The use of a feed ratio of 4Vp:Ac = 25:75% in the presence of 1:100 molar amount of AIBN produced a copolymer with a stoichiometry of 4Vp₃Ac₁ for the RU based on the integration of the ¹H NMR signals of the pyridine ring and the amide NH₂ protons (Fig.2.5). The latter signals disappear upon addition of a few drops of D₂O into the polymer in DMSO allowing us to determine the fraction of the overlapping signal that is attributable to the amide. The stoichiometry of 4Py₃Ac₁ (Figure 2.6) gives an apparent RU molecular mass of 386.5 Da which was used for the calculation of the mole fractions. The molecular mass of the copolymer prepared herein was estimated to be greater than 30 kDa based on ultrafiltration with a membrane of a molecular-weight-cut-off of 30 kDa. The determination of the RU for the rest of the vinylpyridine and acrylamide containing polymers was determined likewise.

An overall summary of all the synthesized polymers and their deduced RU based on ¹H NMR analysis is given in Table 2.11.
Figure 2.5. (Top) $^1$HNMR spectra of $P_1$ in $d_6$-DMSO before and after the addition of D$_2$O. (Bottom) Comparison of the spectra before and after the addition of D$_2$O and substraction of the two spectra.
Figure 2.6. Example of an average repeating unit present in copolymer P₁ determined by integration of ¹HNMR signals.

In order to determine the repeating units of the polymers that did not contain solvent exchangeable protons, the non-overlapping signals attributable to functional groups present on the polymer were used and were compared to signals due to the pyridine protons. The polymer containing 4-vinylpyridine and styrene with feed ratio 25:75% prepared using 1:100 AIBN gives two ¹HNMR signals at 6.5 and 7.0 ppm which correspond to chemical shifts of the two ortho –protons of 4-vinylpyridine and the five protons of the styrene ring with a total integration of 5.48 (Fig. 2.8). Since the two meta-vinylpyridine protons have an integral equal to one and their integral is equal to the one by the ortho-protons it follows that the fraction of this integral attributable to the styrene portion of the repeating unit is ~4.5 and combining this with the pyridine to styrene proton ratio of 2 to 5 we can conclude that the repeating unit of the polymer is ~1 vinylpyridine to 2 styrene.

Likewise, the RU of the copolymer containing vinylpyridine and methyl– methacrylate (MMA) was determined by comparing the integration of the ortho-vinylpyridine signals
to the methylester -CH$_3$ protons visible at 2.8 ppm which reflect the ratio of methylvinylacetate to vinylpyridine (Fig. 2.9) The copolymer containing 2-vinylpyridine and acrylamide (P$_4$) has four peaks in the $^1$HNMR spectra are they are due to the pyridine (6.74, 7.06, 7.48, 6.75 ppm) and the NH$_2$ protons of the amide which overlap with the pyridine signals (Fig.2.10). Since the latter are solvent exchangeable, they disappear by addition of D$_2$O therefore by comparing the integration of these two peaks before and after the addition, the fraction of the peaks due to the amide is reflected in the decrease of the integration.
Figure 2.7. $^1$HNMR spectra of 4VP: Styrene ($P_{30}$) in $d_6$-DMSO. The integration of the proton signals is shown underneath each signal. The proton signals used for determination of repeating unit and the signals of interest are shown.
Figure 2.8. $^1$HNMR spectra of $P_{20}$ (4Vp: MMA, top) and $P_{1a}$ (4Vp:Aa, bottom) containing polymers in d$_6$-DMSO.
Figure 2.9. $^1$HNMR spectra of P$_4$ in d$_6$-DMSO before and after the addition of D$_2$O
<table>
<thead>
<tr>
<th>Copolymer/Feed ratio</th>
<th>Symbol</th>
<th>Average RU</th>
</tr>
</thead>
<tbody>
<tr>
<td>4VP:AM /25:75%</td>
<td>P_1</td>
<td>3 pyridines to 1 amide</td>
</tr>
<tr>
<td>4VP:AM /50:50%</td>
<td>P_8</td>
<td>4 pyridines to 1 amide</td>
</tr>
<tr>
<td>4VP:AM /75:25%</td>
<td>P_9</td>
<td>5 pyridines to 1 amide</td>
</tr>
<tr>
<td>2VP:AM /25:75%</td>
<td>P_4</td>
<td>3 pyridines to 1 amide</td>
</tr>
<tr>
<td>2VP:AM /50:50%</td>
<td>P_5</td>
<td>4.5 pyridines to 1 amide</td>
</tr>
<tr>
<td>2VP:AM /75:25%</td>
<td>P_6</td>
<td>7.4 pyridines to 1 amide</td>
</tr>
<tr>
<td>4VP:MMA /25:75%</td>
<td>P_{20}</td>
<td>2 pyridines to 1 MMA</td>
</tr>
<tr>
<td>4VP:STY /25:75%</td>
<td>P_{30}</td>
<td>1 pyridines to 2 STY</td>
</tr>
<tr>
<td>4VP:AA /25:75%</td>
<td>P_{1a}</td>
<td>3 pyridines to 1 AA</td>
</tr>
</tbody>
</table>

**Table 2.16.** Average repeating units (RU) of synthesized polymers based on ratios of $^1$H NMR integrations of functional groups.
2. Functional group determination using $^{13}$CNMR and HMQC

The identity of some of the synthesized polymers was verified by $^{13}$CNMR. The polymer with repeating unit Vp$_3$Am$_1$ (P$_1$) was characterized more intensively due to its high reactivity in the catalytic reactions studied in this dissertation, therefore the $^{13}$CNMR, COSY, HMQC, and $^1$HNMR were obtained. The incorporation of both of the monomers in the synthesized polymer was verified by the appearance of signals attributed to the pyridine and the amide functional groups (see section 1 in this chapter). Furthermore, the $^{13}$CNMR spectra (Fig. 2.12) contain peaks at 40, 125, 150, 153 and 175 ppm. The first peak matches the methyl or general aliphatic peaks consistent with the aliphatic carbons of the polymer backbone, while the 125 is consistent with the carbonyl carbon. The remaining signals are due to the carbons on the 4-vinylpyridine ring. The correlation of the signals in the HMQC (Fig. 2.11) for the 2−vinylpyridine and acrylamide copolymer also shows that the signals in the 6−8 ppm window of the $^1$H NMR are primarily due to the 2−vinylpyridine ring protons even though the amide signals are overlapping in the same region. The COSY spectra for P1 also verify that both the pyridine and the amide monomers are incorporated on the resulting polymer (Fig. 2.13).
Figure 2.10. HMQC spectra of polymer P4 (2Vp: Am).
Figure 2.11. $^{13}$C NMR spectra of P1 in $d$-6-DMSO
Figure 2.12. COSY spectra of P1 in d$_6$-DMSO
B. Transition metal binding to synthesized polymers

1. Metal binding stoichiometry by use of Co(II) as a probe

The metal binding capabilities of P1 (three pyridines to one acrylamide repeating unit) was further characterized using Co(II) as a probe. A solution of 0.5 mM P1 (concentration calculated based on repeating unit molecular weight of 386.5) was prepared in d6−DMSO and the 1H NMR spectra obtained on a 500 MHz NMR using a 5 mm triple resonance probe. A solution of CoCl2 (50 mM) was prepared in d6−DMSO and two equivalents of Co(II) based on the polymer concentration where added in the polymer solution resulting in the spectra shown in Fig. 2.13 (diamagnetic region) and Fig. 2.15 (paramagnetic region). The two signals appearing in the paramagnetic region are attributed to pyridine binding to Co(II) in solution. A decrease of the integration of the pyridine signals in the diamagnetic region (6.8 and 8.3 ppm) shown in Fig. 2.14 is due to the metal binding to the pyridine ring through the nitrogen. These signals do not change in intensity after addition of 2 equivalents of Co(II) to P1.
Figure 2.13. $^1$HNMR spectra showing the paramagnetically shifted signals of the pyridine protons. The signals are observed after addition of two equivalents of Co(II) to a 0.5 mM sample of P$_1$ in d$_6$-DMSO.
**Figure 2.14.** $^1$HNMR spectra of P1 before addition of 2.0 equivalents of Co(II) in $d_6$-DMSO (diamagnetic region). The integration of protons is shown underneath each signal.
Figure 2.15. $^1$HNMR spectra of P1 after addition of 2.0 equivalents of Co(II) in d$_6$-DMSO. The integration of the signal at 8.3 ppm decreases reflecting the binding of the pyridine to the Co(II) added in solution.
2. IR and UV–vis of synthesized polymers and their metal derivatives

The IR spectra of copolymer P1 was obtained in KBr (1 mg was ground in 200 mg dry KBr) and the IR spectra was obtained on a Nicolet 380 FT–IR instrument. The spectra was compared with the IR spectra of the Fe(III)-P1 complex obtained using the same procedure (Fig. 2.17). The pyridine ring has two stretching bands at 1450 cm\(^{-1}\) and 1487 cm\(^{-1}\) which can be attributed to the C=N stretch and the amide group gives peaks at 1670 cm\(^{-1}\) and 1600 cm\(^{-1}\) due to the C=O stretching. These peaks change upon metal binding to the polymer. A new peak appears at 1637 cm\(^{-1}\) and the previous peaks shift to 1637 and 1603.9 cm\(^{-1}\) respectively, which supporting potential interaction of the metal with the carbonyl group. Furthermore, the C=N stretch changes to higher wavenumber which supports the binding of the metal to the pyridine ring.

An optical titration was also performed using a solution of FeCl\(_3\)6H\(_2\)O (0.1 mM solution prepared in methanol) and titrating different equivalents of 0.1 mM P1( based on the repeating unit) in methanol. The metal was gradually titrated to the polymer solution while the UV–vis spectra of the resulting solution was recorded. The spectral features of the polymer changed in the UV region upon addition of the metal, with a shift of the \(\lambda_{\text{max}}\) peak of the metal, until 1.2 equivalents of the polymer was added. Taken together, these experiments suggest that Fe(III) binds to the polymer and the stoichiometry is approximately 1:1 for Fe(III) to polymer P1 repeating unit.
Figure 2.16. IR spectra of P1 (top) and Fe(III)-P1 (bottom) in KBr.
Figure 2.17. Optical titration of 1mM Fe(III) into 1 mM P1 solution in methanol.
IV. Conclusion and Perspectives

Free radical polymerization was the main method for the synthesis of copolymers in this dissertation. This method possesses many attractive features, such as applicability for a wide range of polymerizable groups, including styrene, vinyl, acrylic, and methacrylic derivatives, as well as tolerance to many solvents, small amounts of impurities, and various functional groups present in the monomers. However, classical free radical polymerization has some limitations as far as its application to the preparation of well-defined macromolecules, as a result it is difficult to control the identity of the repeating unit since the amount of monomer incorporated into the chain is related to their activity (see Q−e scheme section 2). Moreover, the reactivity of the propagating free radical chains and the tendency to undergo a variety of termination reactions are also the drawbacks. As a result, the ending products are polydispersed with very limited control over molecular weight and architecture. One of the ways these limitations of free radical polymerization has been addressed is by the development of ionic polymerizations (anionic or cationic) which allows for efficiency in control of structure and architecture of vinyl polymers and enabled the synthesis of more complex macromolecular architectures (Figure 2.3).

Although these techniques ensure the preparation of materials with low polydispersity, controlled molecular weight, and defined chain ends, they are not useful for the polymerization and copolymerization of a wide range of functionalized vinylic monomers. This limitation is due to the incompatibility of the growing polymer chain end (anion or cation) with numerous functional groups and certain monomer families. Furthermore, these polymerization techniques require strictly controlled reaction
conditions including the use of ultrapure reagents and the strict exclusion of water and oxygen.

New methods of free radical polymerization developed to overcome the drawbacks of ionic polymerization are collectively referred to as “controlled” free radical polymerization. These methods use reagents that reversibly transform the propagating radicals into a dormant species. Thus, the polymer chains grow simultaneously in stead of one after the other. As a consequence, chain growth and monomer consumption take place at a comparable rate. The processes that have received most attention are:

i) nitroxide-mediated radical polymerization (NMRP),

ii) atom transfer radical polymerization (ATRP) catalysed by transition metal complexes, and

iii) polymerization via reversible addition-fragmentation chain transfer (RAFT) or macromolecular design via interchange of xanthates MADIX in which xanthates are used as chain transfer agents.

The dissertation project aimed at identifying the type of functional groups that are more suitable for the formation of the most active catalyst. The metal-binding vinyl pyridine and acrylamide being possibly involved in H-bonding interactions seem to fit the need. The synthesis of the polymers containing pyridine and acrylamide can be pursued in the future by the use of new polymerization techniques mentioned above to better control the molecular weight distribution and the polydispersity of the material.

The use of these methods as means for the generation of polymeric ligands for the construction of metallopolymers can improve control over the structure of the resulting catalysts and significantly narrow the polydispersity of the material. Moreover, a new
generation of copolymers containing pyridine and other functional groups can be generated by further chemical modification of the copolymer, such as a simple hydrolysis of $\text{P1}$ with three pyridines and one acrylamide in the repeating unit to yield $\text{P1a}$ with pyridine and acrylic acid as shown in this dissertation which can further undergo modification via esterification, amide formation, and others, thus allowing for the design of a new generation of copolymers with the same monomer stoichiometry without the need to synthesize polymers from vinyl monomer starting materials. In addition, amides are susceptible to modification by reaction with aryl halides where the aryl group can be attached to the product and the amide is cleaved off. In this way, the repeating unit ratio of pyridine to functional side group can be preserved, allowing for a direct evaluation of the effect of side groups on catalytic activity with the metal binding environment kept constant. Taken together, $\text{P1}$ copolymer prepared herein can serve as a versatile polymeric ligand for future preparation of other analogous copolymers that are useful for further investigation of structure-function correlation of metallopolymers.
References


3661-3688.


CHAPTER 3. HYDROLYTIC ACTIVITIES OF Fe(III) AND Cu(II) DERIVATIVES OF SYTHESIZED COPOLYMERS AND COMMERCIALY AVAILABLE RESINS TOWARDS PHOSPHOESTER HYDROLYSIS*

I. Introduction

Water activation comprises the main pathway through which bond cleavage is achieved in degradative mechanisms of important macromolecules in vivo. For example, the destruction of foreign DNA and proteins, degradation of native proteins to generate their corresponding amino acids, mutations in native DNA that must be removed and repaired, removal of phosphate in dephosphorylation which can modulate protein activity and ATP hydrolysis for energy generation are tasks achieved through water activation and are generally known as hydrolytic reactions (Figure 3.1). Hydrolysis of phosphoester bonds and the reverse reactions of phosphorylation are key steps in biological signaling, which trigger many normal and disease-related metabolic pathways.\(^1\) Hydrolysis is also involved in many important industrial, and environmental processes,\(^2\) and it is considered a cleaner alternative in efforts to eliminate pesticides and nerve agents, including the so-called G-agents (Sarin and Salman) and the VX-agent.\(^3\)

During hydrolysis, the nucleophilicity of a water molecule must increase. Since the pK\(_a\) of unactivated water is very high (15.74), the strong nucleophilic hydroxide cannot

be generated under neutral pH. Such a task is achieved by use of enzymes which can aid in water activation through a general base provided by protein residues or through coordination of the metal center in the active site. Thus, the water molecule becomes substantially more acidic compared to free water molecules and as a result hydrolysis can be possible under physiological conditions. In a metal-centered hydrolysis, the pKₐ of a coordinated water molecule is dramatically lowered by the metal center of high Lewis acidity that is a key factor for the observed activity. Apart from aiding the generation of nucleophile, the substrate can be significantly polarized by the metal center upon binding and becomes more susceptible to nucleophilic attack.

![Figure 3.1. Hydrolysis of peptide and phoshoester bonds](image)

The role of metal ions in hydrolytic reactions is well exemplified by their presence in active sites of enzymes which catalyze the cleavage of phosphoester and peptide bonds (Figure 3.1). The kinetic stability of these bonds is unparalleled, having hydrolytic half lives far exceeding biological timescales. The half life for the hydrolysis of phosphodiester bonds in DNA at neutral pH and 25 °C is estimated to be 130,000 years.
<table>
<thead>
<tr>
<th>Family</th>
<th>Enzyme</th>
<th>metal</th>
<th>Hydrolytic Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidases</td>
<td>Carboxypeptidase</td>
<td>Zn$^{2+}$</td>
<td>C-terminal peptide hydrolysis</td>
</tr>
<tr>
<td></td>
<td>Aminopeptidase</td>
<td>2 Zn$^{2+}$</td>
<td>N-terminal hydrolysis</td>
</tr>
<tr>
<td></td>
<td>Dipeptidase</td>
<td>Zn$^{2+}$</td>
<td>hydrolysis of dipeptides</td>
</tr>
<tr>
<td></td>
<td>Neutral Protease</td>
<td>Zn$^{2+}$/Ca$^{2+}$</td>
<td>hydrolysis of peptides</td>
</tr>
<tr>
<td>Nucleases</td>
<td>Ribonuclease H</td>
<td>2Mg$^{2+}$(Mn$^{2+})$</td>
<td>hydrolysis of RNA (single stranded in RNA-DNA)</td>
</tr>
<tr>
<td></td>
<td>EcoRV Endonuclease</td>
<td>2Mg$^{2+}$</td>
<td>DNA hydrolysis (double stranded)</td>
</tr>
<tr>
<td></td>
<td>P1 Nuclease</td>
<td>3Zn$^{2+}$</td>
<td>DNA, RNA (single stranded)</td>
</tr>
<tr>
<td>Collagenases</td>
<td>Collagenase</td>
<td>Zn$^{2+}$</td>
<td>Collagen hydrolysis</td>
</tr>
<tr>
<td>Phospholipases</td>
<td>Phospholipase C</td>
<td>3Zn$^{2+}$</td>
<td>Phospholipid hydrolysis</td>
</tr>
<tr>
<td>Phosphatases</td>
<td>Alkaline phosphatase</td>
<td>2Zn$^{2+}$/Mg$^{2+}$</td>
<td>Alkylphosphates</td>
</tr>
<tr>
<td></td>
<td>Purple acidphosphatase</td>
<td>Fe$^{2+}$, Fe$^{3+}$</td>
<td>Alkylphosphates</td>
</tr>
<tr>
<td></td>
<td>β-Lactamase</td>
<td>Zn$^{2+}$</td>
<td>Hydrolysis of β-lactam</td>
</tr>
<tr>
<td>Lactamases</td>
<td>β-Lactamase</td>
<td>Zn$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>ATPases</td>
<td>ATPase</td>
<td>Mg$^{2+}$</td>
<td>ATP hydrolysis</td>
</tr>
<tr>
<td>Hydratases</td>
<td>Nitrile hydratase</td>
<td>Fe$^{3+}$</td>
<td>Nitrile hydration</td>
</tr>
</tbody>
</table>

**Table 3.1.** Classes of metallohydrolases and their biological function.\textsuperscript{6, 7}

while for peptide bonds in proteins is approximately seven years under the same conditions.\textsuperscript{4, 5} This stability is primarily due to the repulsion exerted by the negative
charge on the phosphoester bonds or the delocalization of electron density in peptide bonds. It ensures the integrity of the linkage of nucleic acids and proteins respectively. However, this stability must be overcome when these bonds are hydrolyzed.

The majority of metallohydrolases contain Zn$^{2+}$ in the active center, a preference that has been attributed to its high Lewis acidity and fast ligand exchange. Iron(III) centers are not common in active sites of hydrolytic enzymes, but they are best know to be involved in oxidation and/or oxygenation chemistry and to mediate electron transfer, such as ribonucleotide reductase, mono- and di-oxygenases, peroxidases, and cytochromes$^8$. The action of some DNA-binding and cleaving antibiotics is also known to be dependent upon Fe$^9$ such as the anthracyclines and bleomycin. Nevertheless, the established role in metallohydrolases$^{10}$ such as the dinuclear Fe centers in purple acid phosphatase$^{11}$ with non-heme Fe(III)Fe(II) or Fe(III)Zn(II) center (Figure 3.2) which catalyzes the non-specific hydrolysis of phosphomonoesters and the mononuclear center nitrile hydratases$^{12}$ with a low spin Fe(III) in the active site catalyzing the hydration of nitriles, suggests that the high Lewis acidity of Fe$^{3+}$ is consistent with the hydrolytic and hydration activities of these enzymes.

Numerous chemical models have been synthesized for the exploration of biological metal-centered chemistry on the basis that a mono- or dinuclear metal-binding sites can be formed and a specific recognition site may be present. This investigation has provided much insight into the mechanism of metal-centered hydrolysis. The metals typically employed in such model systems include Co$^{3+}$, Cu$^{2+}$, Zn$^{2+}$, and lanthanides(III). Despite the very high Lewis acidity of Fe$^{3+}$ and extensive investigation of Fe-centered oxidation/oxygenation chemistry, hydrolysis by Fe complexes has been much less
explored with only few examples of Fe containing complexes exhibiting significant hydrolytic\textsuperscript{13} and oxidative DNA cleavage activities\textsuperscript{14} suggesting that various Fe complexes should be further explored for hydrolytic catalysis. Some of the characteristic examples of model complexes in literature are summarized in Table 3.2.

\textbf{Figure 3.2}\textsuperscript{11} Proposed mechanism for phosphoester hydrolysis at the kidney bean purple acid phosphatase Fe(II)Zn(II) site.
Most catalytic hydrolyses are typically conducted in homogeneous solutions to mimic the biological environment. While homogeneous catalysis is efficient and convenient, it suffers from difficulty in recovery of the catalyst and incorporation into a recycling or continuous flow process. These difficulties can be overcome by immobilizing catalytic centers on the surface of different matrices. Despite extensive use in many types of reactions, heterogeneous catalysis has not been widely applied to hydrolytic processes. Nevertheless, some recent investigations demonstrated the efficacy of heterogeneous catalysis for hydrolytic reactions. In this chapter, the investigation of several polymer-bound hydrolytic catalysts is described.

### Table 3.2. Mononuclear and dinuclear model complexes catalyzing phosphoester hydrolysis at pH 7.0

<table>
<thead>
<tr>
<th>Homogeneous Catalysts(^{15a-d})</th>
<th>Substr.</th>
<th>(k_{\text{cat}}) (\text{s}^{-1})</th>
<th>(K_m) (M)</th>
<th>(T^\circ) (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{en}]_2\text{Co}^{3+}-\text{OH}]^{2+})(^{a})</td>
<td>BNPP</td>
<td>(3 \times 10^{-5})</td>
<td>–</td>
<td>50</td>
</tr>
<tr>
<td>([9]\text{Cu-OH}^+)(^{b})</td>
<td>BNPP</td>
<td>(5 \times 10^{-7})</td>
<td>–</td>
<td>25</td>
</tr>
<tr>
<td>([\text{Zn}_2(\text{BPAN})(\mu-\text{OH})(\mu-\text{O}_2\text{Ph}_2)]^{2+})(^{c})</td>
<td>BNPP</td>
<td>(4.1 \times 10^{-6})</td>
<td>(2 \times 10^{-3})</td>
<td>25</td>
</tr>
<tr>
<td>(\text{Cu}_2(\text{H}_2\text{bbppnol})(\mu-\text{oac})(\text{H}_2\text{O})_2\text{Cl}_2)(^{d})</td>
<td>BNPP</td>
<td>(5.4 \times 10^{-5})</td>
<td>(1.2 \times 10^{-2})</td>
<td>25</td>
</tr>
</tbody>
</table>

Abbreviations: \([9]\) = 1,4,7-triazacyclononane; \(\text{en}\) = ethylenediamine;

\(\text{BPAN} = 2,7-\text{bis}[2-(2-\text{pyridylethyl})-\text{aminomethyl}]\text{–}1,8-\text{naphthyridin}\)

\(\text{H}_2\text{bbppnol} = \text{N}, \text{N'},\text{N},\text{N'}-\text{bis}[2-\text{hydroxylbenzyl}(2-\text{pyridylmethyl})]-2-\text{ol–}1,3-\text{propane diamine}\)
<table>
<thead>
<tr>
<th>Homogeneous Catalysts</th>
<th>Substr.</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$T^\circ$ (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(vbpy) (polymer)$^{16b}$</td>
<td>BNPP</td>
<td>$2.3 \times 10^{-5}$</td>
<td>25</td>
</tr>
<tr>
<td>Cu(adenine) (polymer)$^{16c}$</td>
<td>BNPP</td>
<td>$2.23 \times 10^{-6}$</td>
<td>30</td>
</tr>
<tr>
<td>Cu(P1)(polymer)$^{15a}$</td>
<td>BNPP</td>
<td>$8.3 \times 10^{-6}$</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>BNPP</td>
<td>$8.3 \times 10^{-6}$</td>
<td>25</td>
</tr>
</tbody>
</table>

Abbreviations: vbpy = vinylbipyridine

Table 3.3. Kinetic parameters for heterogeneous hydrolysis of BNPP at pH 8.0 by mononuclear polymeric complexes.

II. Experimental: Materials and Methods

The copolymers P1 (three pyridines one acrylamide repeating unit), P1a (three pyridines one acrylic acid), P9 (five pyridines and one acrylamide) were synthesized following the procedure described in Chapter 2. The buffers HEPES, CAPS, MOPS and sodium acetate, chelex and the substrates bis(p-nitrophenyl) phosphate (BNPP), p-nitrophenyl phosphate (NPP), p-nitrophenylphenyl phosphonate (NPPP), tris(p-nitrophenyl)phosphate and Leu-p-nitroanilide from Sigma-Aldrich (St Louis, MO). The buffer solutions were treated with Chelex resin to remove any trace amount of metal ions. The resins Tris-(2-
aminomethylamine)-styrene divinylbenzene bound and diethylenetriamine-styrene DVB bound were purchased from Aldrich.

1. Preparation of copolymers: The copolymer of 4-vinylpyridine (4Vp) and acrylamide (Ac) with an average repeating units (RU) of $4Vp_3Ac_1$ ($P_1$) was prepared as described in chapter 2. The stoichiometry of $4Py_3Ac_1$ gives an apparent RU formula mass of 386.5 Da which was used for the calculation of the concentration of polymer solutions.

The use of a 4Vp:Ac ratio of 3:1 under the same conditions for the preparation of $P_1$ above affords a copolymer ($P_9$) with stoichiometry of $4Vp_5Ac_1$ for its RU based on $^1H$ NMR. A copolymer ($P_{1a}$) of 4Vp and acrylic acid (Aa) with stoichiometry of $4Vp_3Aa_1$ for its RU can be prepared directly from $P_1$ by base-hydrolysis with 0.5 M NaOH solution, and verified with $^1H$ NMR to reveal the disappearance of the solvent exchangeable amide NH$_2$ signals.

2. Preparation of metal complexes of copolymers: The copolymers mentioned in section 2 readily bind Fe$^{3+}$ in methanol. The resulting complexes are insoluble brown metallopolymers precipitate which is active towards phosphoester hydrolysis. The optimum metal-to-polymer repeating unit stoichiometry for the catalysis was determined by titrating 1.0 mM polymer with Fe$^{3+}$ in methanol solution and checking the hydrolytic activity of the resulting insoluble complex by monitoring the hydrolysis of 1.0 mM BNPP.

3. Computer modeling: Molecular mechanics calculations of the metal-binding mode using the MM3 protocol were performed by the use of BioMedCAChe version 6.1.10 (Fujitsu, Beaverton, Oregon). The coordination geometry of the metal is set to be octahedral, and the metal is considered to bind to pyridine in-plane with the pyridine ring.
Any distortion from this coordination chemistry is considered not to be a favorable metal-binding mode.

4. Kinetic measurements: The initial rates of the hydrolysis of the substrates were monitored spectrophotometrically on a Cary 50 (Varian) or an Ultrospec1000 (Pharmacia Biotech) within 2–5 hours through the change at 405 nm due to the production of 4-nitrophenolate ($\varepsilon = 17,500 \text{ M}^{-1} \text{ cm}^{-1}$). A typical experiment was conducted by preparing 1.0 mM P1 (repeating unit molecular weight 386.5) in methanol–buffer HEPES at pH 8.0 and mixing 1:1 volumes of Fe(III) and P1 to obtain 0.4 mM Fe(III)–P1 complex. A stock solution of 20 mM of phosphodiester substrate was prepared and was aliquots were added into the reaction medium in order to obtain substrate concentrations in the range of 0.5–10.0 mM.

Due to the insolubility of the catalyst to the reaction medium, the catalysis is heterogeneous and the amount of product is determined after centrifuging the reaction mixture to separate the supernatant for optical measurement at certain time interval. Various amounts of the complex and the substrates were used in the reactions, from which rate laws were determined and rate constants obtained. Fe$^{3+}$ ion (which forms precipitates as rust under the experimental conditions) and copolymer solutions were used as the controls and showed negligible activity when compared to the Fe$^{3+}$-P1 complex.

5. Oxidative cleavage of plasmid DNA: The oxidative cleavage of plasmid DNA was observed following separation by 1% agarose gel electrophoresis (150 V, 1× Tris-acetate buffer for one hour), stained with ethyldium bromide and then photographed on a transilluminator.
A volume of 10.0 µL of Fe^{3+}-P1 (21.0 µM of Fe^{3+} and 3.8 µM P1 repeating unit in solution), 0.8 µL of 150 ng/µL plasmid DNA (0.12 µg of plasmid DNA in solution) and 2.0 µL of 1% H$_2$O$_2$ (0.15 % or 43.5 mM in solution) was incubated for several different time points. The reference samples contained the same concentrations of all the reagents but no polymer. All DNA cleavage assays were buffered with 100 mM HEPES at pH 8.0. The experiment was repeated three times to ensure consistency. All plasticware was demetallized with EDTA and washed several times with 18.2 M-Ω-cm water.

## III. Results

1. Hydrolytic activity of Fe$^{3+}$- P1

   A. Metal binding and catalyst stoichiometry

   The pyridine and acrylamide-containing copolymer family has been previously determined to bind various metal ions.$^{11}$ The copolymer P1 with a stoichiometry of 4Vp$_3$Ac$_1$ as RU readily binds Fe$^{3+}$ in methanol to give an insoluble brown metallopolymer precipitate. The activity of Fe$^{3+}$-P1 toward the hydrolysis of 1.0 mM bis(p-nitrophenyl) phosphate (BNPP), a prototypical phosphodiester substrate, was determined as a function of its [RU] under pseudo first-order conditions in 1:1 buffer:methanol at pH 8.0 and 25 °C, from which a rate constant $k_{obs}$ of $2.92 \times 10^{-6}$ s$^{-1}$ was obtained.

   In order to determine the optimum metal-to-polymer stoichiometry for catalysis, a 1.0-mM polymer (methanol: buffer HEPES 25mM pH 8.0) mixture was titrated with
methanol solution of Fe\(^{3+}\) and the resulting heterogeneous solution was checked for activity towards hydrolysis of 1.0-mM BNPP at pH 8.0 and 25 °C. A linear increase in activity with Fe\(^{3+}\) addition was observed until one equivalent of metal ion to polymer RU was added (Fig. 3.3), indicating a 1:1 stoichiometry of metal binding necessary for optimum catalysis. Fitting of the activity with respect to the amount of metal gives an apparent formation constant of 49,000 M\(^{-1}\) for Fe\(^{3+}\)-P1. To ensure that the observed activity is due to the insoluble metallopolymer complex, the heterogeneous Fe\(^{3+}\)-P1/solvent mixture was centrifuged and the solid and the supernatant were evaluated for activity towards hydrolysis of 1.0-mM BNPP.
Figure 3.3. Titration of Fe$^{3+}$ into 1.0 mM RU of copolymer 4Vp$_3$Ac$_1$, monitored with the activity toward the hydrolysis of 1.0 mM BNPP in 50% methanol solution of 25 mM HEPES at pH 8.0 at 25º C. The solid trace is the fitting to a 1:1 complex formation.
The activity of the supernatant was negligible while the activity of the solid matched that observed for the initial heterogeneous solution, indicating a heterogeneous catalysis. The catalyst can be easily recovered into a recycling or continuous flow process. By immobilizing catalytic centers on the surface of different matrices,\(^1\) as in the iron-polymer system present herein. Despite its extensive use in many types of reactions, heterogeneous catalysis has not been widely applied to hydrolytic processes\(^2\) and should be further explored.

To further confirm the metal-to-polymer stoichiometry of the metallopolymer that exhibits the hydrolytic activity, a Job plot\(^2\) was constructed which used optical absorption to determine metal to ligand stoichiometry. Since the Fe\(^{3+}\) complex of the copolymer is not soluble, we have modified the optical Job plot into an “activity Job plot” in which the activity toward the hydrolysis of BNPP instead of the optical density of the complex was determined with respect to the mole fraction of Fe\(^{3+}\) (\(X_{\text{Fe}}\)) or polymer RU (\(X_{\text{RU}} = 1 - X_{\text{Fe}}\)) at a constant total concentration \([\text{Fe}^{3+}] + [\text{RU}]\) of 2.0 mM. The maximum in the activity Job plot is found at \(X_{\text{RU}} - X_{\text{M}} \approx 0.5\), indicating that the predominant active species is iron-polymer with a stoichiometry of

\[X_{\text{Fe}^{3+}}: X_{\text{RU}} = 1:1\] which is consistent with the stoichiometry obtained from the titration of Fe\(^{3+}\) to polymer (Fig. 3.3).
Figure 3.4. ‘Activity Job Plot’ of Fe$^{3+}$-4Vp3Ac$_1$ in which the initial rate of hydrolysis of 1 mM BNPP is plotted against $X_{Fe}$ at a constant total concentration of [RU]+[Fe$^{3+}$] of 2.0mM. The reaction pH is maintained at pH 8.0 in HEPES buffer in 50 methanol at 25º C. The solid trace is the fitting of the data to a metal–ligand binding equilibrium where the total [Fe] + [P1] is constant and equal to 2 mM.
B. Kinetic characterization of phosphoester hydrolyses

The plot of the initial rate for the hydrolysis of 1.0 mM BNPP by various amounts of the complex of [Fe$^{3+}$-RU] is linear (Fig. 3.5) which affords an observed rate constant $k_{\text{obs}}$ of $4.5 \times 10^{-6}$ s$^{-1}$ for the rate law $\text{rate} = k_{\text{obs}}[\text{Fe}^{3+}\text{-RU}]$. Although BNPP has been generally acknowledged to be quite accessible to hydrolysis due to the very good leaving group $p$-nitrophenol, its auto hydrolytic rate is still extremely low with a rate constant $k_0 = 1.1 \times 10^{-11}$ s$^{-1}$ at pH 7.0 and 25 °C, i.e., $1.1 \times 10^{-10}$ s$^{-1}$ at pH 8, considering OH$^-$ as the nucleophile. Thus, the hydrolysis of BNPP by Fe$^{3+}$-P1 shows a significant rate enhancement of $4.1 \times 10^4$ with respect to the non-catalyzed hydrolytic reaction of BNPP under the same conditions.

In order to establish the rate law for the heterogeneous hydrolysis of the above substrates by Fe$^{3+}$-P1 complex, the initial rates for the hydrolysis of the above substrates were determined in a range of 0.2–4.0 mM of the few substrates above at 25 °C and pH 8.0. To ensure efficient binding of the metal under all the experimental conditions during the kinetic studies (particularly at high pHs wherein metal ions can easily precipitate out as hydroxides), an excess amount of the polymer (RU/Fe$^{3+} \sim 2.5$ in terms of equivalent) is added into the reaction. The rate with respect to substrate concentration [S] is found to be hyperbolic (Fig. 3.5), indicating that the hydrolysis probably follows pre-equilibrium kinetics similar to enzyme catalysis. This kinetics can be described as the binding of the substrate S to the metal center to form an intermediate P1-Fe$^{3+}$-S complex, followed by conversion of the bound substrate to the products Eq.1 (below). The rate law for this
Figure 3.5. (Top) Initial rate of hydrolysis of 1 mM BNPP by different amounts of Fe(III)--P1 at pH 8.0. (bottom) Plot of the initial hydrolytic rates of PNP(■), BNPP(▲) and NPPP(●) by 0.8 mM [Fe$^{3+}$-RU] of the 4Vp$_{3}$Ac$_{1}$ copolymer at pH 8.0 and 25º C. The data represent the average of three runs and the solid curves represent the best fit to a pre-equilibrium rate law (Eq.(2)).
reaction can be obtained with steady-state approximation and an assumption that the amount of bound S is much less than amount of free S in solution, which is expressed as Eq.(2) in which \( K' = \frac{k_{-1} + k_{\text{cat}}}{k_{1}} \) is the virtual dissociation constant of the bound S. The data can be fitted to Eq. (2), to give the rate constants \( k_{\text{cat}} \) (under pseudo-first order conditions of high \([S] \gg K'\)) and \( K' \), and a second-order rate constant \( k_{\text{cat}}/K' \) at low \([S] \ll K'\). The good fitting verifies the pre-equilibrium pathway for the catalysis shown in Eq.(1), indicating direct substrate binding with the metallopolymer. The highest rate constants are obtained for the phosphodiester BNPP with a first-order rate constant \( k_{\text{cat}} \) of \( 5.6 \times 10^{-6} \text{ s}^{-1} \) and a second-order rate constant \( k_{\text{cat}}/K' \) of 0.0125 M\(^{-1}\) s\(^{-1}\), affording a significant catalytic proficiency of \( 5.6 \times 10^{4} \) in terms of the first-order rate constant and \( 6.3 \times 10^{9} \) in terms of the second-order rate constant with respect to the rate constants of the uncatalyzed hydrolysis.

\[
\text{Fe}^{3+}-\text{P1} + \text{S} \xrightleftharpoons[k_{-1}]{k_{1}} \text{P1-Fe}^{3+}-\text{S} \xrightarrow{k_{\text{cat}}} \text{Fe}^{3+}-\text{P1} + \text{Prod.} \quad \text{Eq.1}
\]

\[
\text{rate} = \frac{k_{\text{cat}}[\text{Fe - RU}][S]}{K' + [S]} \quad \text{Eq.2}
\]

In addition to this phosphodiester substrate, \( \text{Fe}^{3+}-\text{P1} \) is also active toward the hydrolysis of a phosphomonoester \( p \)-nitrophenyl phosphate (NPP) and a phosphonate ester \( p \)-nitrophenylphenyl phosphonate (NPPP) (Fig. 3.5 and Table 3.6), but not the phosphotriester tris\((p \)-nitrophenyl\)phosphate (TNPP) and the peptide mimic Leu-\( p \)-nitroanilide under the same experimental conditions. The order of reactivity for the substrates is BNPP >> NPPP > NPP in terms of their rate constants. However, the auto-
hydrolytic rate of these phosphoesters is in the order of NPP ~ NPPP >> BNPP (Table 3.6), indicating a specific selectivity of Fe$^{3+}$-P1 toward phosphodiesters.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Catalyst</th>
<th>$k_{cat}$/ s$^{-1}$</th>
<th>$K'$/ mM</th>
<th>$k_{cat}/K'$ (s$^{-1}$ M$^{-1}$)</th>
<th>catalytic proficiency</th>
<th>half life</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNPP</td>
<td>None</td>
<td>$1.1 \times 10^{-10}$</td>
<td></td>
<td></td>
<td></td>
<td>203 yr</td>
</tr>
<tr>
<td>BNPP</td>
<td>Fe$^{3+}$-P1</td>
<td>$(5.63 \pm 0.64) \times 10^{-6}$</td>
<td>0.45 $\pm$ 0.07</td>
<td>0.0125</td>
<td>$5.1 \times 10^{4}$</td>
<td>1.5 d</td>
</tr>
<tr>
<td>NPPP</td>
<td>None$^{16}$</td>
<td>$7.65 \times 10^{-8}$</td>
<td></td>
<td></td>
<td></td>
<td>3.5 mon</td>
</tr>
<tr>
<td>NPPP</td>
<td>Fe$^{3+}$-P1</td>
<td>$(1.47 \pm 0.08) \times 10^{-5}$</td>
<td>0.95 $\pm$ 0.12</td>
<td>0.0154</td>
<td>193</td>
<td>13 h</td>
</tr>
<tr>
<td>NPP</td>
<td>None$^{17}$</td>
<td>$8.2 \times 10^{-8}$</td>
<td></td>
<td></td>
<td></td>
<td>3.3 mon</td>
</tr>
<tr>
<td>NPP</td>
<td>Fe$^{3+}$-P1</td>
<td>$(9.53 \pm 0.11) \times 10^{-7}$</td>
<td>0.12 $\pm$ 0.03</td>
<td>$7.94 \times 10^{-3}$</td>
<td>12</td>
<td>8.5 d</td>
</tr>
</tbody>
</table>

**Table 3.4.** Summary of catalytic constants for the hydrolysis of phosphoesters.

Conditions: reaction volume = 2.5 mL, T = 25 °C, pH = 8.0 (HEPES buffer 25 mM), [Fe$^{3+}$] = 0.8 mM, [BNPP]=0.3-6.0 mM, [PNPP]=0.13-2.5 mM, [NPPP]=0.5-10.0 mM.
C. Effect of pH on rate of phosphoester hydrolysis by Fe(III)--P1

The effect of pH on the activity of Fe$^{3+}$-P1 is investigated using BNPP as a substrate to reveal the status of the nucleophile. This study is of interest since hydrolysis is in general more favorable at high pHs, whereas Fe$^{3+}$ ions form hydroxide and coagulates at pHs greater than 2.0. Hydrolysis of BNPP by Fe$^{3+}$-P1 was studied in the pH range of 4.5-10.0, affording a bell-shape profile (Fig. 3.6). Fitting of the data to an apparent two-ionization process reveals two ionization constants $pK_1 = 5.3$ and $pK_2 = 8.6$. The first ionization is attributable to a nucleophilic water molecule coordinated to the Fe$^{3+}$ in the complex because there is no other group that is expected to ionize around neutral pH. The low $pK_a$ value reflects the strong Lewis acidity of the Fe$^{3+}$ center, like in the case of Fe$^{3+}$ containing purple acid phosphatase. The second $pK_a$ is possibly associated with the formation of iron hydroxide precipitate determined to be catalytically inactive in buffer solution at pH 8.0 which is prominent at higher pHs due to the small $K_{sp}$ value ($2.6 \times 10^{-39}$) of Fe(OH)$_3$. The small $K_{sp}$ value indicates that 1.0 mM Fe$^{3+}$ would form Fe(OH)$_3$ at pH as low as ~2.1. The difference between this value and $pK_2$ reflects that Fe$^{3+}$ is tightly bound to the polymer with an intrinsic affinity constant of $\sim 3.2 \times 10^6$ M$^{-1}$ (i.e., $10^{-2.1}/10^{-8.6}$), 70 times higher than the apparent binding affinity estimated from metal-activity titration at pH 8.0 which may not reveal the affinity constant well owing to the large magnitude. The results obtained herein are consistent with a metal-centered mechanism for the hydrolysis of BNPP by this metallopolymers. However, whether the catalysis is mononuclear or multinuclear cannot be concluded from the results shown here. Nevertheless, the linear increase in activity with the addition of Fe$^{3+}$ before reaching the end point reflects the lack of cooperativity between two metal centers.
**Figure 3.6.** Effect of pH on the hydrolysis of BNPP. The data are fitted to a two-ionization process according to the equation $V = V_{\text{lim}} / (1 + [H^+] / K_a)(1 + K_b / [H^+])$, in which the ionized form is the only active form, $V$ the hydrolytic rate, $V_{\text{lim}}$ is the rate for the deprotonated active species and $K_a$ the ionization constant.
D. Catalyst recycling

One advantage of heterogeneous catalysts is their easy separation and recycling. To demonstrate that the Fe$^{3+}$-P1 catalyst is also reusable due to its heterogeneous nature, the catalyst is recovered by centrifugation after a typical reaction toward substrate in the range of 1–4 mM, washed repeatedly with buffer and methanol, and then added to freshly prepared solutions of BNPP for activity determination. The recycled Fe$^{3+}$-P1 complex is able to continuously catalyze the hydrolysis of BNPP for three cycles (Figure 3.7). The recycling after 3 times shows a small loss of its activity, attributed to that the recovered catalyst is ~20% less in mass after the extensive recycling due to its powdery nature.
Figure 3.7. Recycling experiments for the hydrolysis of 1 mM (A), 2 mM (B) and 4 mM BNPP (C) by 0.8 mM Fe(III)-P1 at pH 8.0 in 50% methanol. Cycle 1 (●), cycle 2 (○) and cycle 3 (●).
E. Molecular mechanics calculations

Molecular mechanics calculations (MM3 force field) were performed on a short piece of polymer of three RU, which reveal that the metal is bound to PI polymer via pyridines preferably from adjacent RU’s separated by 3–5 side chains, such as one pyridine side chain at position I (or II) of one RU and the second at any position (or II–IV) in the next RU to afford a total energy of around –200 kcal/mol for the different binding modes (Fig.3.8). Binding of metal to pyridines less than this separation apart causes the coordination to significantly distort from an octahedral coordination and the coordinated pyridine ring to pucker. The third ligand can come from the subsequent RU (Fig. 3.8, top) or from an adjacent polymer chain (Fig. 3.8, bottom). The coagulation of the polymer upon metal binding suggests a possibility that the polymer chains tangle together upon metal binding, likely resulted from inter-stand cross linkage through metal binding as in the latter binding mode. The MM3 calculations on the latter cross-strand metal binding also reveal that this binding mode is the most stable form with a total energy of –640 kcal/mol.

The binding of the substrate BNPP is also modeled in the MM3 calculations. The saturation kinetics described above reflects that the substrate is bound to the active site (the metal) first before it is hydrolyzed. The binding of one oxygen of the phospho-center to the metal affords a configuration with the coordinated nucleophilic water (solid red sphere) situated at the trans position of the leaving group about 3.2 Å away from the phosphorus center. Moreover, the amide group in this metallopolymer seems to serve as a general base to further activate the coordinated water and/or as a H-bond donor/acceptor to stabilize the bound substrate during the catalysis (particularly in the
case of the inter-strand metal binding; Fig. 4, bottom), thus further stabilizing the bound substrate to assist the hydrolytic activity.

**Figure 3.8.** Molecular mechanics calculations of the metal-binding mode using the MM3 protocol (Top) The substrate BNPP is modeled into the catalytic site with Fe(III) bound by a total of three pyridines (each pyridine comes from different repeating units) (b) Interaction of BNPP phosphate with the amide of the polymer chain.
F. Polymer design

To provide more insight into the role of the amide functional group present in the repeating unit of the copolymer \( P_1 \) toward hydrolytic activity, the copolymers \( 4Vp_5Ac_1 \) (\( P_9 \)), \( 4Vp_3Aa_1 \) (\( P_1a \)), and polyvinylpyridine (\( P_0 \)) were also used as potential ligands for the formation of \( Fe^{3+} \)-polymer complexes and their hydrolytic activities checked (Table 3.5). These copolymers contain functional groups other than amide, thus their \( Fe^{3+} \) complexes are expected to exhibit different activities from \( Fe^{3+} \)-\( P_1 \) toward the hydrolysis of BNPP. The activities of \( P_9 \) with slightly higher content of pyridine shows hydrolytic activity comparable to \( P_1 \) while the \( P_0 \) shows similar rates to \( Fe^{3+} \)-\( P_1 \) than an order of a magnitude higher activity towards BNPP hydrolysis. The \( k_{cat} \) values of BNPP hydrolysis by \( Fe^{3+} \)-\( P_1a \) is about 10 folds lower than that by \( Fe^{3+} \)-\( P_1 \), whereas the second-order rate constant \( k_{cat}/K' \) of BNPP hydrolysis by \( Fe^{3+} \)-\( P_1a \) is nearly four orders lower than that by \( Fe^{3+} \)-\( P_1 \) at pH 8.0 which may be attributed to possible repulsion between the negatively charged BNPP and the acrylate functional group of the copolymer as reflected by the much larger virtual dissociation constant in the latter case (19.3 and 0.45 mM respectively, Table 3.5).

The structures of \( Fe^{3+} \)-\( P_1 \) and \( Fe^{3+} \)-\( P_1a \) differ by an amide functional group. The amide group is suspected to be involved in H-bonding with the substrate based on MM3 calculations (Fig. 3.8). If this H-bonding interaction is the sole difference between \( Fe^{3+} \)-\( P_1 \) and \( Fe^{3+} \)-\( P_1a \) for stabilizing the transition state during the hydrolysis, the lack of such H-bond in the former case would increase the activation energy by about 20 kJ/mol for an average H-bond energy which would decrease the \( k_{cat} \) value by about 3000 times. The \( k_{cat} \) values for \( Fe^{3+} \)-\( P_1 \) and \( Fe^{3+} \)-\( P_1a \) differ by only 37 times, indicating that there should
be other interactions that are also involved in transition-state stabilization in addition to the H–bonding interaction associated with the amide group, such as possible involvement of a water molecule for the interaction between the carboxylate group and the phosphoester substrate.

![Graph](image)

**Figure 3.9.** Comparison of saturation profiles for BNPP hydrolysis catalyzed by 0.8 mM Fe(III) complexed to polymers of different repeating units. P9 (●) has repeating unit of five pyridines to one amide, P1a (▽) contains three pyridines and one acrylic acid, P1(▼) contains three pyridines to one amide and polyVP(○) is polyvinylpyridine (we assume the repeating unit to be four pyridines). The activity is monitored at pH 8.0 in 50% methanol.
<table>
<thead>
<tr>
<th>Polymer ligand</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K'$ (mM)</th>
<th>$k / K'$ M$^{-1}$ s$^{-1}$</th>
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</thead>
<tbody>
<tr>
<td>P1 (Vp$_3$: Am$_1$)</td>
<td>$5.6 \times 10^{-6}$</td>
<td>0.45</td>
<td>$1.24 \times 10^{-2}$</td>
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<td>P9 (Vp$_5$:Am$_1$)</td>
<td>$4.3 \times 10^{-6}$</td>
<td>0.39</td>
<td>$1.1 \times 10^{-2}$</td>
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<tr>
<td>P1a (Vp:Am)</td>
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<td>19.3</td>
<td>$7.8 \times 10^{-6}$</td>
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<tr>
<td>Po (polyVp)</td>
<td>$7.1 \times 10^{-6}$</td>
<td>1.73</td>
<td>$4.1 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

**Table 3.5.** Catalytic constants for Fe$^{3+}$ complexes of various copolymers. The fitting data are shown in Figure 3.9. above.
G. Inhibition of BNPP hydrolysis by HPO$_4^{2-}$

Since the phosphoesters used as substrates are hydrolyzed by the Fe(III)–P1 complex with small virtual dissociation constant $K'$ for a bound phosphodiester, phosphomonoester and phosphonate, it would be informative to evaluate the binding of a simple phosphate center to the complex. Dibasic phosphate is utilized here, which is an ultimate hydrolytic product of phosphoesters. Phosphate can significantly inhibit the rate of hydrolysis of 1.0 mM BNPP by Fe$^{3+}$-P1 with an IC$_{50}$ of 100 µM (Fig. 3.10). The observed inhibition was analyzed to follow a mixed type of inhibition (i.e., different $K'$ and $k_{cat}$ values at different inhibitor concentrations, Fig. 3.11) with an inhibition constant of 4.9mM. Such inhibition pattern cannot be simply due to binding of phosphate to the metal center, but may be possibly attributed to binding and removal of the metal from the polymer by phosphate. To verify this hypothesis, the Fe$^{3+}$-P1 complex was isolated by centrifugation after a typical reaction in the presence of 0.1mM phosphate (Fig. 3.10). The solid was washed with 1M calcium chloride to remove traces of substrate, and then rinsed thoroughly. After addition of one equivalent of Fe$^{3+}$ and saturating amounts of BNPP under typical reaction conditions, the activity to the solution was checked and it matched the previously observed activity under the same conditions. This result confirms that the inhibition can be due to removal of the metal from the active complex. It is also indicative that the decreased efficiency of the hydrolysis of phosphomonoester PNP as compared to the phosphodiester BNPP hydrolysis may be partially due to the significant product inhibition by phosphate. Since a phospho-center is demonstrated to interact with Fe$^{3+}$-P1, it is interesting to investigate whether or not the phosphodiester bond in DNA can interact with this metallopolymer and whether or not cleavage can occur.
Figure 3.10. Inhibition of 1.0 mM BNPP hydrolysis by HPO$_4^{2-}$ in the presence of 0.8 mM Fe$^{3+}$-P1 at pH 8.0 (25 mM). The data represent triplicate experiments.
**Figure 3.11.** Lineweaver-Burk Plots for BNPP hydrolysis at 0 (□), 0.01 (●) and 0.02(○) and 0.2(∇) mM HPO$_4^{2-}$ in the presence of 0.4 mM Fe(III)–P1 at pH 8.0 in HEPES buffer 25 mM and 50% methanol.
H. Oxidative plasmid DNA cleavage

Supercoiled and circular forms of the plasmid are clearly seen as revealed in the reference cell (Fig.3.12, lane 2). Nicking of the supercoiled plasmid by Fe$^{3+}$-P$_9$ occurs rapidly within 10 min at room temperature in the above reaction conditions with concomitant appearance of linearized plasmid (center band) and increase in intensity of the relaxed nicked circular form (top band). The increase in intensity of the nicked circle and linear bands suggests that the appearance of the linearized form does not exclusively follow a sequential mechanism where the nicked product is further cleaved to the linearized form. The observed cleaving pattern in the reaction course suggests that the linearized form can be directly produced by double strand cleavage of the supercoiled plasmid. This in fact can be considered to be the predominant cleaving pathway as supported by the persistence of the nicked circular band after ~2 hours of reaction.

Comparison of the plasmid cleaving pattern of Fe$^{3+}$-P$_9$ with the pattern of equivalent amount of Fe$^{3+}$ under the same conditions reveals that the observed reaction is not due to action of free metal ions which lead to exclusive formation of nicked circular plasmid in the same time frame (Fig.3.12)
Figure 3.12. Cleavage of plasmid DNA by Fe$^{3+}$-P9 in the presence of 0.12 µg of plasmid DNA, 0.15 % H$_2$O$_2$, 21.0 µM of Fe$^{3+}$ 3.8 µM P1 RU (lanes on right) and DNA by Fe$^{3+}$ without polymer (lanes 4-9) in the presence of 0.12 µg of plasmid DNA, 0.15 % H$_2$O$_2$ and 21.0 µM of Fe$^{3+}$ in 100 mM HEPES at pH 8.0. Lane 2 contains 0.12 µg of plasmid DNA and 0.15 % H$_2$O$_2$ in 100 mM HEPES buffer at pH 8.0.
2. Heterogeneous hydrolysis catalyzed by metal complexes of crosslinked resins

The commercially available cross-linked polystyrene divinylbenzene resins containing iminodiacetate (—N(CH₂COO)₂, diethylenetriamine (resin 1) and tris-(2-aminomethyl)amine (resin 2) functional groups can readily bind 2⁺ and 3⁺ transition metal ions to form neutral and positively charged complexes, respectively (Figure 3.13). The latter is expected to have high Lewis acidity suitable for hydrolytic catalysis. The Fe³⁺ complexes of each resin is prepared by adding slightly excess amount of freshly prepared 100 mM methanol solution of FeCl₃ to resin suspended in methanol. The excess unbound Fe³⁺ can be removed by filtration and extensive washing with methanol, then washed with buffer for immediate use or air-dried for long-term storage. The Cu²⁺ and Zn²⁺ complexes of the resins are prepared similarly, using aqueous solutions of the corresponding metal salts. A direct metal titration determines the metal-binding capacity of the Chelex resin to be 1.97 meq/g, 0.72 meq/g for resin 1 and 0.19 meq/g for resin 2 consistent with the specification provided by the manufacturer (2.0 meq/g for the chelex, 4.40 mmol N/g and 2.64 mmol N/g for resins 1 and 2 respectively). The metal binding capacity of resins 1 and 2 was experimentally determined through direct metal titration to be 0.72 meq/g and 0.19 meq/g respectively.

Phosphoester hydrolysis is carried out by suspending a fixed amount of Fe³⁺-Resin in 4 mL of a substrate solution in 100 mM TRIS buffer at pH 9.0 under constant stirring. The initial rate of the hydrolysis is determined spectrophotometrically based on the release of the chromophore p-nitrophenol (ε₄₀₅ = 1.91 × 10⁴ M⁻¹cm⁻¹ at pH 9.0) from the substrate in the supernatant at different time.
Figure 3.13. Structures of resins used in the heterogeneous hydrolytic reactions.

From top: Chelex resin, Resin 2 (middle) and Resin 1 (bottom).
intervals, and subtract the background hydrolysis using metal-free resin as the control. All hydrolytic reactions were performed four times and the average and the standard deviation determined. The initial rates for the hydrolysis of the substrates at different pHs are obtained similarly in appropriate buffer solutions of 100 mM. The “apparent concentration” of Fe-Chelex in the solution is expressed as the molar amount of Fe per unit volume of the solution, i.e., 49.3 mM for 100 mg of the complex in 4 mL of the reacting solution. The concentration of BNPP used for the determination of the rate law is in the range of 0.50−10.0 mM.

The Cu$^{2+}$, Zn$^{2+}$, and Fe$^{3+}$ complexes of Chelex-resin are completely insoluble in common solvents and in the buffers used for the kinetic analysis, thus representing a heterogeneous catalysis in nature. The model phosphodiester substrate bis-(p-nitrophenyl)phosphate (BNPP), the phosphomonoester p-nitrophenyl phosphate, and p-nitrophenyl phenylphosphonate were used to evaluate the efficiency and specificity of the metal complexes of the resin towards hydrolysis of phosphoester bonds. The hydrolysis of BNPP by 100 mg Fe$^{3+}$-Chelex complex is readily observable at ambient temperature of 27 °C and pH 9.0 (Figure 3.14), with a rate of (2.2 ± 0.8) × 10$^{-4}$ mM min$^{-1}$ (i.e., a specific activity of 2.1 × 10$^{-3}$ μmol min$^{-1}$ mg$^{-1}$) for the hydrolysis of 10-mM BNPP. Moreover, the Cu$^{2+}$ and Zn$^{2+}$ complexes of Chelex resin were also found active, yet with much slower rates of 1.3 × 10$^{-5}$ and 1.1 × 10$^{-5}$ mM min$^{-1}$ toward the hydrolysis of 10-mM BNPP under the same conditions as the iron complex. The rate of BNPP hydrolysis increases with increasing amount of Fe$^{3+}$-Chelex complex (Figure 3.14, inset), from which an observed first-order rate constant $k_{obs}$ is obtained to be 5.42 × 10$^{-8}$ s$^{-1}$. The
significantly higher activity of the Fe\textsuperscript{3+} complex suggests that Fe-centered hydrolysis should be further explored, despite the greater attractiveness of Cu\textsuperscript{2+} and Zn\textsuperscript{2+} complexes in hydrolytic chemistry. The reasonably fast catalysis by the Fe\textsuperscript{3+} complex allows an extensive kinetic investigation to be performed.

**Figure 3.14.** The hydrolysis of BNPP (■, 1 mM; ●, 4 mM; and ▲, 10 mM) by 100-mg Fe\textsuperscript{3+}–Chelex complex in 100 mM TRIS buffer at pH 9.0 relative to the uncatalyzed reaction (◆, 1 mM and ▼, 10 mM) under the same conditions. The activity of a typical recycled Fe-Chelex complex is also shown (○). Initial rates are obtained as the slope of the lines.
Figure 3.15. Plot of the initial hydrolytic rate of BNPP at different concentrations by 100-mg Fe(III)-Chelex resin at pH 9.0 and 27 °C. The solid trace is the best fit to the pre-equilibrium kinetics (Eq. 1). The inset shows the initial rate for the hydrolysis of 10-mM BNPP by different amounts of Fe(III)-Chelex resin, from which an observed first-order rate constant $k_{obs}$ can be obtained.
In order to establish the rate law for the heterogeneous hydrolysis of BNPP by Fe$^{3+}$-Chelex complex, the rate is further determined at various [BNPP] values at ambient temperature of 27 °C and pH 9.0. The rate is found to be non-linear with respect to [BNPP] (Fig. 3.15), suggesting an enzyme-like pre-equilibrium kinetics. Such kinetics can be described as the binding of the substrate BNPP (S) to the catalytic center on the surface of the resin, followed by the conversion of the bound substrate into the products (Eq.1, in section 3.3.1 B). The rate law for this reaction can be expressed as Eq.2 (in section 3.3.1 B) by means of steady-state approximation.

The data can be well fitted to that rate law to give a first-order rate constant $k_{\text{cat}} = 1.8 \times 10^{-7}$ s$^{-1}$, $K' = 6.7$ mM, and a second-order rate constant $k_{\text{cat}}/K'$ of $2.7 \times 10^{-5}$ M$^{-1}$ s$^{-1}$ at [S] $<< K'$ to give the rate law $rate = k_{\text{cat}}/K' [\text{Che-Fe}][\text{BNPP}]$ (Figure 3.14). This second-order rate constant is comparable to those of homogeneous hydrolysis of BNPP catalyzed by some simple metal complexes. For example, second-order rate constants are found to be in the range of $(0.18–2.8) \times 10^{-5}$ M$^{-1}$ s$^{-1}$ calculated from corresponding pseudo-first order rate constants at pH 8.36 and 55 °C$^{22}$ and $(5.4–11.5) \times 10^{-5}$ M$^{-1}$ s$^{-1}$ at pH 10.9–11.5 and 35 °C$^{23}$ for several mono- and dinuclear Zn$^{2+}$ complexes; and $(6.0–80) \times 10^{-5}$ M$^{-1}$ s$^{-1}$ at pH 7.2 and 35 °C for Zn$^{2+}$ and Cu$^{2+}$ complexes of cyclic triamines.$^{24–25}$ The auto-hydrolytic rate $k_0 = 2.06 \times 10^{-9}$ s$^{-1}$ of BNPP at 27 °C and pH 9.0 can be extrapolated from directly measured rates at higher temperatures and pH 7.0, assuming OH$^{-}$ is the nucleophile.$^{24}$ The catalysis of BNPP hydrolysis by Fe$^{3+}$-Chelex exhibit a significant catalytic proficiency that is 87-fold higher than auto-hydrolysis in terms of the first-order rate constant expressed as $k_{\text{cat}}/k_0^{25}$, which represents a shortening of the half-life from ~20 years to ~80 days for $rate = k_{\text{cat}}[\text{Che-Fe}]$ at high [S], and $9.8 \times 10^{5}$ times higher in
terms of the second-order rate constant expressed as \((k_{\text{cat}}/K')/(k_0/55.5)^{26}\) for \(rate = (k_{\text{cat}}/K')[\text{Che-Fe}][\text{BNPP}]\) at low [S].

The Fe\(^{3+}\)-chelex can also catalyze the hydrolysis of a phosphonate ester, \(p\)-nitrophenyl phenylphosphonate (PNPP). A rate of \(1.3 \times 10^{-4} \text{ mM min}^{-1}\) was determined at pH 9.0 and ambient temperature with 100 mg of the complex, representing a small rate enhancement of \(\approx 9\) fold against the uncatalyzed rate of \(1.4 \times 10^{-5} \text{ mM min}^{-1}\). Moreover, the Fe\(^{3+}\)-Chelex complex discriminates against the hydrolysis of the phosphomonoester \(p\)-nitrophenyl phosphate, showing negligible rate enhancement under the same experimental conditions. The low activity towards phosphomonoester hydrolysis in this mononuclear iron center might be attributed to the decrease in the Lewis acidity and/or the positive charge of the metal center due to the negatively charged acetate groups on the ligand which results in less effective neutralization of the negative charge on the monoester for the subsequent nucleophilic attack by the coordinated OH\(^-\).

In order to determine the catalytic mechanism, the effect of pH on the hydrolysis of BNPP by the Fe\(^{3+}\)-Chelex complex has also been investigated. A sigmoidal rate–pH profile is revealed (Fig. 3.16), which can be fitted to a single-proton ionization process by considering that the ionized form is the active species. A pK\(_a\) value of 8.8 is obtained from the fitting. That the activity is attributable to a single ionization process is consistent with a hydrolytic mechanism mediated by a metal-bound nucleophilic OH\(^-\).

The relatively high pK\(_a\) value than those of many other metal complexes reflects a relatively low Lewis acidity of the metal center in Fe\(^{3+}\)-Chelex, which might be due to the negative charge on the ligand. It is also noteworthy that the Fe\(^{3+}\) is not removed out from the resin at neutral and higher pHs, a condition that favors the formation of iron rust.
due to the low p$K_a$ of Fe(OH)$_6^{3+}$ which shows negligibly small hydrolytic activity toward BNPP under the experimental conditions.

**Figure 3. 16.** Activity-pH profile for the hydrolysis of 10 mM BNPP in the presence of 100 mg Fe$^{3+}$–Chelex complex. The data are fitted to a single-ionization process according to the equation $rate = V_{\max} / (1 + [H^+] / K_a)$, in which the ionized form is considered the only active form, $K_a$ the ionization constant, and $V_{\max}$ the rate of the fully ionized form.
The hydrolytic rate constants of the copper derivatives of resins 1 and 2 towards BNPP where determined under the same conditions as in the BNPP saturation experiment performed for Fe$^{3+}$ Chelex resin. The catalytic activity for these polymeric complexes was in the same range as those observed for the chelex resin complex (Table 3.6). This finding suggests that the Lewis acidity of the metal center is not the only determining factor in the observed catalytic activity, since that would make the metal containing amine resins (1 and 2) stronger Lewis acids compared to the Fe$^{3+}$-Chelex resin complex.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substrate</th>
<th>$K_{\text{cat}}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(II)-Chelex</td>
<td>BNPP</td>
<td>$3.25 \times 10^{-6}$</td>
</tr>
<tr>
<td>Tris(2-amino)ethylamine-Cu(II)</td>
<td>BNPP</td>
<td>$6.74 \times 10^{-6}$</td>
</tr>
<tr>
<td>Diethylenetriamine-Cu(II)</td>
<td>BNPP</td>
<td>$2.89 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

**Table 3.6.** Summary of the hydrolytic activities of Cu(II) complexes of different resins.
IV. Concluding remarks and future perspectives

In this chapter, the catalytic activity of Fe(III) complexes of cross-linked resins and linear copolymers have been examined toward the hydrolysis of activated phosphoesters. Model complexes of Fe(III) for hydrolytic reactions are not common, despite the high Lewis acidity of Fe(III). To my knowledge, these two systems are the only examples of Fe(III) complexes of polymer ligands that are used as catalysts for phosphoester hydrolysis. Moreover, the findings discussed in this dissertation suggest that the catalytic efficiencies of the cross-linked resins are not very high compared to the efficiencies obtained for the linear copolymer P1, suggesting that cross-linking of the polymeric material may contribute to the decrease in flexibility of the metal centers which could have a negative effect on the efficiency of the catalyst. Both the Fe(III) complex of the Chelex® resin (iminodiacetate functional group) and Fe(III) complex of P1 showed selectivity towards phosphodiester hydrolysis with rate accelerations approaching 1,000 for the cross-linked resins (at pH 9.0) and $5.0 \times 10^4$ for the linear copolymer P1 at pH 8.0. However, the physical characteristics of the cross-linked resins are well suited for large scale catalytic applications. One of the objectives of this project is to identify material that are most suitable for large scale applications, therefore the evaluation of their ease of recycling should be considered along with their catalytic proficiencies. Moreover, metal bound Chelex® resin and diethylenetriamine functionalized resin (Resin 2) used in this study are well suited for large scale applications allowing for a continuous flow system to be developed.
To further test the effect of the cross-linking on the hydrolytic activity, functionalized resins containing pyridine and amide can be prepared and their catalytic efficiencies checked. A synthetic approach toward this end would be to chemically modify the amine containing resins used in this dissertation (section 3.4) through coupling with a pyridine derivative such as 2-chloromethylpyridine. If the resulting material exhibits activity comparable to the hydrolytic activity by Fe(III)−P1, it can be a useful catalyst in large applications of phosphoester and phosphonate ester hydrolysis.

One common use of cross-linked resins is in protein purification where columns are packed with a functionalized resin material capable of interacting with proteins through ionic bonds or allowing separation based on size. An appropriate buffer is pumped through the column aiding in the elution and collection of the fractions separated from the column. Future investigations may include not only catalysis, but specific recognition which may have broad application in biochemistry and medicine such as purification targeted proteins and removal of pathogenic proteins.
References


23, 697–762.


CHAPTER 4. OXIDATION AND HYDROXYLATION ACTIVITIES OF Cu(II) COMPLEXES OF SYNTHESIZED COPOLYMERS TOWARDS (POLY)PHENOLS

I. Introduction

One important use of dioxygen in aerobic organisms, apart from the four-electron reduction to water necessary in respiration shown below, is to function as a source of oxygen atoms in biosynthesis of various biological precursors.¹

\[
\text{O}_2 + 4\text{H}^+ + 4e^- \rightarrow 2\text{H}_2\text{O} \quad E^\circ = + 0.185\text{V}
\]

These biosynthetic processes are catalyzed by a group of enzymes known as monooxygenases or dioxygenases, depending on the number of oxygen atoms incorporated into the final product. Aerobic organisms draw considerable advantage by controlling the oxidizing power of dioxygen and efficiently converting it to a form capable of being stored and used. Oxygen activation must take place during these processes not only in the aforementioned biological applications but also in numerous established synthetic reactions that make use of the activating abilities of oxygenation on inactive hydrocarbons, leading to important synthetic intermediates. Although O₂ is readily available in the atmosphere, its use as an oxidation agent in multi-cell organisms requires transportation, storage, and activation specifically carried out by different
proteins and enzymes that employ metal ions such as Fe-and Cu found in oxidases or oxygenases.

Copper ion is known for its ability to aid in stoichiometric and catalytic oxidative transformations apart from its well established and central role in proteins involved in a diversity of functions such as mixed function oxidases, dioxygen transferases and electron transfer oxidases (Table 4.1). A good example of copper-containing enzymes carrying out this type of activation include di-Cu tyrosinase and polyphenol oxidases.

Copper proteins can be classified into three types based on the different structural and spectroscopic characteristics of their active sites. The type-1 copper centers are also called “blue copper centers” which are typically found in electron-transfer proteins such as plastocyanin and azurin. They are named as such due to their characteristic deep blue color caused by an intense Cys S-to -Cu(II) charge-transfer transition with molar absorptivity \( \varepsilon > 2000 \text{ cm}^{-1} \text{M}^{-1} \). Studies of the crystal structures of blue copper proteins indicate that the metal centers have a strongly distorted tetrahedral coordination sphere and differ up to 22° from the ideal tetrahedron angle. Such characteristic distortion is observed in azurin with a minimal change upon reduction of copper(II) to copper(I), and it is believed to corresponds to an intermediate state between the favored coordination geometries of Cu(II) (square-planar) and Cu(I) (trigonal). The “non-blue copper centers” form the class of type-2 centers and are found in oxidases such as galactose oxidase and in oxygenases such as dopamine-b-monooxygenase. In addition, a type-2 copper center appears in the dinuclear metal site of Cu,Zn-superoxide dismutase. Type-2 copper centers are characterized by a tetragonally distorted coordination sphere and they exhibit the spectroscopic behavior of copper(II) known from the Cu(II) hexaaqua complex.
In contrast to the mononuclear copper centers, the type-3 copper centers contain two copper ions. The copper proteins belonging to the type-3 group play an important role in the biological transport, storage, and activation of dioxygen. Tyrosinase, catechol oxidase, and hemocyanin belong to this group of proteins and have similar spectroscopic characteristics. They all have two copper ions coordinated to three histidines each (Figure 4.1). Hemocyanins (HC) are a group of oxygen carrier proteins and can be divided into two classes depending on their biological sources: the arthropodan (lobsters and spiders) and the molluscan (octopus and snails) hemocyanins. They have different subunit organization and their evolutionary relationship is weak. Hemocyanin has been crystallographically characterized in both the reduced and oxygenated forms. In both forms of the protein there is a tris (histidine) coordination at each copper center. In the reduced state, the two trigonally coordinated Cu(I) ions are separated by 4.5 Å, and in the oxy-state O$_2$ is reduced by 2e$^-$ to form the peroxide ion which is coordinated in a bridging and side on $\mu$-$\eta^2$: $\eta^2$ configuration. The spectroscopic similarity of oxy-Tyrosinase and oxy-Catechol oxidase with oxy-Hemocyanin suggests their similar coordination environments in all three oxygenated forms.

Some copper proteins e.g., laccase, ascorbate oxidase, and ceruloplasmins cannot be classified in any of the above categories. The copper sites in these proteins include a trinuclear copper moiety often described as a combination of a type-2 and a type-3 center. In ascorbate oxidase, a type-1 copper center is about 13 Å distant from such a trinuclear copper unit. Yet two other types of copper sites Cu$_A$ and Cu$_B$ are found in cytochrome c oxidase, which also has several non-copper metal centers.
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<th>Function</th>
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<td>hemocyanin</td>
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<td>tyrosinase</td>
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<td>Oxidation</td>
<td>cytochrome c oxidase</td>
<td>$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$</td>
</tr>
<tr>
<td></td>
<td>galactose oxidase</td>
<td>$2\text{XH}_2 + \text{O}_2 \rightarrow 2\text{X} + 2\text{H}_2\text{O}$</td>
</tr>
<tr>
<td></td>
<td>amine oxidase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ascorbate oxidase</td>
<td>$\text{XH}_2 + \text{O}_2 \rightarrow \text{X} + \text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td>Superoxide dismutation</td>
<td>Cu-Zn SOD</td>
<td>$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$</td>
</tr>
</tbody>
</table>

*Table 4.1. Copper proteins involved in dioxygen metabolism.*
Tyrosinase (EC 1.14.18.1) is one of the first enzymes to be discovered as an oxygenase based on $^{18}\text{O}_2$ labeling experiments carried out by H. S. Mason et. al in 1955. The hydroxylation of tyrosine yields the neurologically significant DOPA (dihydroxyphenylalanine), which is then further oxidized by tyrosinase or polyphenol oxidase to give $o$-dopaquinone and ultimately forms melanin pigments. It can also catalyze the oxidation of monophenols in air under reduction conditions to $o$-diphenols (catechol) and the oxidation of $o$-diphenols to $o$-quinones (Fig. 4.1). Tyrosinase has also been used in biosensors for oxygen and phenol detection, as a catalyst for degradation of pollutant phenols and in selective synthesis of $o$-quinones and related polymers, and in several other applications such as in the cosmetic industry and the food industry. It is also widely distributed among bacteria, fungi and plants.

*Catechol oxidase* is a copper enzyme functionally and structurally related to tyrosinase. It is isolated from plants and is able to catalyze the oxidation of catechol to quinone without exhibiting hydroxylation activities as observed in tyrosinase. The reaction catalyzed by catechol oxidase is important in determination of hormonally active catecholamines dopamine, norepinephrine, and DOPA. The copper centers in isolated catechol oxidases are EPR-silent and have been assigned to an antiferromagnetically coupled Cu(II)-Cu(II) pair. X-ray absorption Spectroscopic investigations on the met form of catechol from *Lycopus europaeus* and *Ipomoea batatas* (Fig. 4.2) revealed that the active site consists of a dinuclear Cu(II) center where the two metals are coordinated by four N/O donor ligands. The short metal-metal distance and the EPR investigations support a $\mu$-hydroxo bridged dicopper(II) active site in the met – form of the protein.
The oxy form of tyrosinase catalyzes the conversion of monophenol to the corresponding quinone through the ortho-diphenol formation. In this scheme, His$^{54}$ is released from the Cu$^A$ site, resulting in the formation of the bidentate intermediate. The met and oxy forms of tyrosinase can catalyze the conversion of ortho-diphenol to the corresponding quinone. This reaction should progress similarly to that of catechol oxidase.
Figure 4.2. Structure of *Ibomonea batatas* Catechol Oxidase (ibCO). Copper ions are shown in orange, α helixes in blue, β sheets in green, and disulfide bonds in yellow.
A detailed catalytic mechanism describing the action for tyrosinase and catechol oxidase has been proposed by Solomon et al (Figure 4.3).\textsuperscript{4,13} The \textit{oxy} state is believed to be the starting point of the hydroxylation activity commencing with the binding of a monophenol substrate and subsequent monoxygenation to form \textit{o}–\textit{diphenol} which then binds to the \textit{met} form of the enzyme in a bidentate mode (Figure 4.3). Oxidation of the diphenol substrate leads to the formation of the reduced dinuclear copper center (\textit{deoxy} state). Binding of dioxygen generates the \textit{oxy} state of the enzyme necessary for completion of the catalytic cycle. The catalytic cycle of the catecholase activity can be initiated by the \textit{oxy} or the \textit{met} states. A diphenol substrate binds to the dinuclear center upon deprotonation followed by oxidation to form the first quinone and the \textit{deoxy} or reduced state of the enzyme which can subsequently bind dioxygen and generate the \textit{oxy} state which is further attacked by a second diphenol molecule. Upon oxidation and formation of the second quinone molecule, the \textit{met} state of the enzyme is generated and the catalytic cycle is complete.

Despite the low sequence identity of tyrosinase with \textit{Ipomoea batatas} catechol oxidase (25.3\%) and the \textit{odg} domain of the \textit{Octopus dofleini} hemocyanin (26\%), the overall active sites are quite similar as revealed by the recently solved crystal structure of \textit{Streptomyces antibioticus} tyrosinase\textsuperscript{14} (See figure 4.4). The positional differences of the main chain atoms between tyrosinase and \textit{Ipomoea} catechol oxidase and \textit{Octopus} hemocyanin are within 1 Å for an average of 150 residues. Furthermore, a high degree of conservation is observed in the catalytic core domains of these three proteins. A common feature in their active sites is the presence of two closely spaced copper ions coordinated by three His residues through N\textsubscript{ε} nitrogen atoms characteristic of type-3 proteins and a
similarity in their ability to bind molecular oxygen. The reason for the different functions exhibited by these proteins is believed to result from a variation in the substrate-binding pocket and the accessibility of the substrate to the active site. The Phe$^{261}$ side chain in the active site of catechol oxidase located above the Cu$^\text{A}$ site (Figure 4.4) partially prevents substrate binding. In contrast, the substrate-binding pocket of tyrosinase has a larger vacant space above the dicopper center. Moreover, a strong correlation between the members of the type-3 class of copper proteins is supported by the weak catecholase activity found for molluscan hemocyanin$^{15}$ anthropodan and tarantula HCs$^{16}$ (in the latter case after partial proteolytic digestion with trypsin or chymotrypsin) and by the phenolase activity ofby plant COs using special monophenols such as 4-hydroxyanisole.$^{17}$ Furthermore, weak catalase activity (dismutation of hydrogen peroxide) has been detected for HC, TYR and CO.$^{18}$ It remains to be shown whether the oxidizing species and their mechanism of action, identified in several model complexes performing hydroxylations, are also present in the case of these proteins and enzymes.
Figure 4.3. Mechanism of cresolase and catecholase activity of tyrosinase and/or Catechol oxidase initially proposed by Solomon and co-workers and including more recent results. Note that the met form is inactive toward phenol oxidation and hydroxylation.
Figure 4.4. The active centers of tyrosinase and of structurally homologous proteins.

14(A) active center of the met form I of tyrosinase complexed with ORF378. Carbon atoms from the residues of tyrosinase and ORF378 are shown in orange and cyan, respectively. (B) active center of the inhibitor-bound potato catechol oxidase. Carbon atoms from catechol oxidase and inhibitor (phenylthiourea, PTU) are shown in orange and purple, respectively. (C) active center of the oxy form of the octopus hemocyanin. Carbon atoms from hemocyanin are shown in orange and the bridging peroxo in red.
1. Model systems for oxygen activation

Copper-dioxygen chemistry is relevant to the utilization of an oxygen donor, mainly O$_2$, hydrogen peroxide and peracids, in oxidative transformation of organic molecules by Cu complexes that mimic the action of Cu-containing oxidases. These biomimetic systems may be more available, stable, and catalytically versatile than enzymes, thus may have wide applications, and can serve as model systems to provide further insight into the mechanisms of the aforementioned enzymes. Design aspects of biomimetic systems rely on the structural features of their biological templates, such as the metal-binding sites in metalloenzymes. The use of model compounds has been crucial in gaining insight into the binding mode of dioxygen to the copper centers of these enzymes.

Major breakthroughs in copper dioxygen chemistry have occurred in crystal structures of three discrete Cu$_2$O$_2$ complexes. In one of these studies, Karlin et. al observed one possible binding mode for dioxygen where it is bound in the peroxide oxidation state as cis-$\mu$-$\eta^1$-$\eta^1$ where O$_2^{2-}$ is a bridging molecule as it was found in a model compound (Figure 4.5). In constrast, Kitajima showed an alternative binding mode where oxygen is bound in the $\mu$-$\eta^2$-$\eta^2$ mode where O$_2^{2-}$ is localized between the two Cu atoms. The latter binding mode was observed in [Cu(HB(3,5-i-Pr$_2$pz)$_3$)$_2$(O$_2$)] and its spectroscopic behavior and magnetism closely resembled those observed in OxyHC. The similarity between this model compound and oxyHC was later supported by X-ray structural analysis of this protein isolated from horseshoe crab Luminus polyphemus, confirming the validity of the $\mu$-$\eta^2$-$\eta^2$ binding mode.
Figure 4.5. Copper dioxygen binding modes\textsuperscript{29} (copper ligands have been omitted)
Further work by Tolman\textsuperscript{28} showed that there exists yet another potential intermediate, found only in model complexes so far, where two Cu(III) ions are held together by oxygen bridges without an O-O bond, in a bis (µ-oxo) configuration found in equilibrium with the µ-η\textsuperscript{2}: η\textsuperscript{2} binding mode. The O-O bond can be present or absent depending on the ligand, solvent, and temperature used.\textsuperscript{29} Much of the literature in this area of research is focused on the spectroscopic characterization of these species and the specificity in the reactions they catalyze.\textsuperscript{30,31} It has been established that more electron donating ligands favor the formation of the bis(µ-oxo) species, and that tetradeionate amine ligands favor formation of the end-on peroxo dicopper (II) compounds while tridentate and bidentate ones can generate both the side-on peroxo and the bis(µ-oxo) dicopper species.\textsuperscript{32} As far as the reactions catalyzed by these intermediates, so far only the µ-η\textsuperscript{2}: η\textsuperscript{2} species has been shown to be involved in endogenous and exogenous hydroxylations of phenolic substrates and catechol oxidation reactions while the µ-η\textsuperscript{2}: η\textsuperscript{2} and the bis (µ-oxo) has been shown to afford endogenous hydroxylation of aromatic ligands.\textsuperscript{33}

Several complexes capable of mimicking the activity of tyrosinase\textsuperscript{30,31} have been crystallized while some model compounds exhibit catecholase and phenolase activity.\textsuperscript{32,33} Overall, most of the literature examples of model compounds are capable of mimicking the catecholase activity rather than the hydroxylation reaction and can achieve turnover numbers approximately 10,000–fold lower than the native enzymes. Some of the most active complexes and their catalytic activities are given in Table 4.2.

It is noteworthy to mention that active complexes can alternatively be prepared by treatment of Cu(II) starting materials with H\textsubscript{2}O\textsubscript{2} or ROOH which can also be formed.
upon oxygenation of Cu(I) form of the complexes (as discussed above). The binding of H$_2$O$_2$ to the di-Cu(II) center is analogous to the “shunt” pathway observed in the case of iron-heme chemistry$^{34}$ Based on this finding, O$_2$ and H$_2$O$_2$ have been used interchangeably for the investigation of the catalytic oxidation and hydroxylation reactions by Cu(II) complexes.

The distinct difference between the mechanism of tyrosinase and catechol oxidase is still under investigation. An area of much focus has been the characterization of the nature of the hydroxylating species and the fate of dioxygen in the reaction. The main question in the hydroxylation mechanism has been whether the cleavage of the O–O bond occurs before, during, or after the attack on the aromatic ring of the substrate. In the first case, the hydroxylation must be mediated by a bis(µ-oxo) core, in the second case it would be an example of electrophilic aromatic substitution by peroxide on the π–system of the phenolic ring activated by the existing –OH group while the last possibility involves the formation of an aryl peroxide which reacts with the quinone (Figure 4.6).

It has been shown that the side–on bound peroxide is the least negative form of peroxide compared to the bis(µ-oxo) core. Based on work performed by Karlin et.al, the hydroxylation of an aromatic π–system occurs via the µ-$\eta^2$: $\eta^2$ intermediate and has been supported by Raman spectroscopic studies and molecular orbital theory.$^{47}$ However, the characterization of the bis(µ–oxo) core in small model complexes and its interconversion with the side–on bridging peroxide, coupled with the observation that the above core is able to hydroxylate aromatic rings (so far of endogenous ligands rather than exogenous
<table>
<thead>
<tr>
<th>Complex</th>
<th>Substrate</th>
<th>Conditions</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{Cu}_2(\text{L}^{1a-e}(\text{ClO}_4))]^{\text{+}}$</td>
<td>Catechol</td>
<td>MeOH, 25$^\circ$C</td>
<td>$5.1 \times 10^{-4}$</td>
<td>41</td>
</tr>
<tr>
<td>$\text{Cu}_2[(\text{L}55)]^{4+}$</td>
<td>DTBC</td>
<td>pH 5.1/MeOH</td>
<td>1.1</td>
<td>46</td>
</tr>
<tr>
<td>$[\text{Cu}_2(\text{PPPNOL})(\text{OO}_3\text{HC})]^{\text{+}}$</td>
<td>DTBC</td>
<td>pH 8.0, 25$^\circ$C</td>
<td>$3.1 \times 10^{-3}$</td>
<td>45</td>
</tr>
<tr>
<td>$[\text{Cu}(\text{L})(\text{NCS})]_2$</td>
<td>DTBC</td>
<td>MeOH</td>
<td>$1.3 \times 10^{-2}$</td>
<td>42</td>
</tr>
<tr>
<td>$\text{Cu}_2(\text{pyrazolate})$</td>
<td>DTBC</td>
<td>MeOH</td>
<td>0.68</td>
<td>43</td>
</tr>
<tr>
<td>$\text{Cu}_2(\text{pyridyl(en)})$</td>
<td>ArOX $\text{X} = \text{halogens}$</td>
<td>Acetone, -94$^\circ$C</td>
<td>0.01-1.0</td>
<td>44</td>
</tr>
<tr>
<td>$\text{Cu}_2(\text{aminocarbohydrates})$</td>
<td>3,5-di-$t$-butylphenol</td>
<td>MeOH, 25$^\circ$C</td>
<td>$5.0 \times 10^{-2}$</td>
<td>39</td>
</tr>
<tr>
<td>$\text{Cu}_2(\text{diimine})$</td>
<td>2,6-di-$t$-butyl-phenol</td>
<td>MeOH, 25$^\circ$C</td>
<td>0.096</td>
<td>38</td>
</tr>
<tr>
<td>$\text{Cu}(\text{II})-\text{A}\beta_{1-20}$</td>
<td>catechol</td>
<td>buffer pH 7.0 25$^\circ$C</td>
<td>0.53</td>
<td>37</td>
</tr>
<tr>
<td>$\text{Cu}_2(\text{DBED})$</td>
<td>phenol</td>
<td>0.21</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>$\text{Cu}_2(\text{R-PYAN})$</td>
<td>DTBC</td>
<td>THF, -80$^\circ$C</td>
<td>30%</td>
<td>36</td>
</tr>
<tr>
<td>$\text{Fe}^{\text{III}}$-porphyrin</td>
<td>DTBC</td>
<td>THF, -78$^\circ$</td>
<td>95%</td>
<td>35</td>
</tr>
</tbody>
</table>

**Table 4.2.** Catalytic parameters for oxidation and hydroxylation reactions catalyzed by some model copper complexes.
Figure 4.6. (Top) Hemocyanin (HC) crystal structure from *Limulus Polyphemus*\(^{48}\).

(Bottom) Kitajima’s complex a bound dioxygen.\(^{26}\)
substrates) provided by W. Tolman, suggests that both of these intermediates can potentially support the electrophilic aromatic substitution as opposed to H abstraction mostly excluded by the absence of deuterium isotope effect.

Moreover, the examples where exogenous substrates rather than endogenous ligand substrates are used in hydroxylations have been limited making these reactions challenging and the design aspects of model complexes subject to an exciting research field.
Figure 4.7. (a) Three possible reaction mechanisms for the oxygenation of phenols by Cu$_2$O$_2$ centers. (b) Reaction pathway for ligand hydroxylation in the model system of Karlin et al. The hydroxylation proceeds via a $\mu$-$\eta^2$: $\eta^2$ intermediate.
2. Hydroxylation of phenolic substrates

Oxygen insertion into unactivated hydrocarbons to yield valuable synthetic intermediates such as alcohols and phenols is a difficult spin-forbidden process. Hydroxylation of aliphatic and aromatic C-H bonds and epoxidation of olefins are thermodynamically favored processes as indicated by the negative enthalpies of reaction. However, the direct reaction with dioxygen in the absence of a catalyst is a very slow process. This kinetic barrier in dioxygen reactions is due to the difference in spin states i.e. the triplet state due to two unpaired electrons of dioxygen and the singlet state of typical organic molecules having no unpaired electrons. Furthermore, the products of oxygenation reactions have singlet ground states. Conversion from triplet to singlet spin state is slower than reactions between molecules as a result the number of unpaired electrons must remain the same before and after each step of such a chemical reaction. For that reason, these reactions (see 4.2 below) cannot take place in a fast and concerted step.

Pathways that obey the spin restriction are all energetically costly and have high energy barriers. For example, the excited triplet states of saturated or unsaturated molecules are at least 40-70 kcal/mol less stable than the ground state and similarly the singlet state dioxygen is 22.5 kcal/mol higher in energy than the ground-state triplet dioxygen.¹

\[ \frac{1}{2} O_2 + ^1X \rightarrow ^1XO \]

\begin{tabular}{c c c}
\hline
\( \overline{\uparrow \uparrow} \) & \( \overline{\uparrow \downarrow} \) & \( \overline{\uparrow \downarrow} \) \\
\hline
\end{tabular}
Table 4.3. Enthalpies of reaction with dioxygen for some hydrocarbons

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔH (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH4(g) + ½ O2(g) → CH3OH(g)</td>
<td>−30</td>
</tr>
<tr>
<td>C6H6(g) + ½ O2(g) → C6H5OH(g)</td>
<td>−43</td>
</tr>
<tr>
<td>CH3H5OH(g) + ½ O2(g) → C6H4(OH)2(g)</td>
<td>−42</td>
</tr>
<tr>
<td>C2H4(g) + ½ O2(g) → C2H4O(g)</td>
<td>−25</td>
</tr>
<tr>
<td>C5H5N(g) + ½ O2(g) → C2H4NO(g)</td>
<td>−13</td>
</tr>
</tbody>
</table>
Mimic of the enzymatic oxidative transformations involving oxygen or hydrogen peroxide activation has been centered in the mechanism of action of heme and non-heme iron catalysts (for cytochrome P450, methane monooxygenase) and copper-centered catalysts (for tyrosinase, catechol oxidase, polyphenol oxidase, laccases). These enzymatic systems owe their catalytic efficiency in a well controlled interaction of the metal center(s) with oxygen or $\text{H}_2\text{O}_2$ to generate a high valent metal oxygen species capable of oxygenation of alkanes (heme/nonheme iron) or a dinuclear center capable of binding of dioxygen and generation of a $\text{Cu}_2\eta^2\text{-O}_2$ center which can perform hydroxylation of phenols and oxidation of catechols to ortho-quinones by a peroxo unit (tyrosinase, catechol oxidase).\textsuperscript{50-53} Understanding the activity and mechanism of these Cu-centered chemical systems can provide information for better understanding of $\text{O}_2$ utilization by copper proteins and enzymes, with possible applications in the fields of environmental green chemistry, aging, cancer research, and oxidative stress-associated apoptosis.\textsuperscript{54-56}

3. Polymer–based systems in copper–centered oxidations

Several metal-binding polymers have recently been prepared and utilized as catalysts for different types of metal-centered reactions.\textsuperscript{57} Polymer based ligands can create an ideal environment for the construction of type-3 copper centers capable of functioning as models of catechol oxidase and tyrosinase due to the ability of the macromolecular environment to mimick these enzymes and provide the proper geometry and distance for the catalytic centers, bringing the copper centers in proximity to facilitate oxygen binding and stabilize the active intermediates. Nevertheless, the literature examples where this
approach has been utilized are scarce. When simple metal complexes are used for the construction of dinuclear copper(II) centers, it is common to use dinucleating macrocyclic ligands as means of keeping the dicopper centers at close proximity while in the case of macromolecular models of dinuclear catalysts, the copper centers can be clustered and the assembly of the dinuclear site can become more facile. In one of the literature examples where the macromolecular approach is used, a hydroxo bridged phenanthroline cupric complex is encapsulated in mesoporous silica using an ion exchange method. The rational of this approach is that the nanochannels of the silica can provide the environment for the two Cu(II) nuclei to maintain the proper distance and configuration for catalytic activity and in part selectivity for the oxidation of di-t-butyl catechol to the corresponding quinone. The heterogeneous material enhanced the activity and selectivity compared to the homogeneous simple complex. Similar effect has been observed by grafting macrocyclic Schiff base ligands on silica via covalent bonds. The resulting dinuclear copper(II) complexes showed significantly higher rate of DTBC oxidation to the corresponding quinone when compared to the homogeneous copper(II) complexes. In another example, a polymeric material based on adenine complexes of copper crosslinked with divinylbenzene is used to construct dinuclear copper centers which has also been shown to able to hydroxylate 4-hydroxyanisole and oxidize catechol and DTBC to the corresponding quinones.

All the above examples essentially utilize a solid support as means of enhancing the stability of a pre-formed complex. Furthermore, in most instances the mechanistic details of the observed catalysis are not discussed or probed in detail. To further explore the versatility of metallopolymers as potential catalysts and biomimetic model systems, the
copolymers of 4-vinyl pyridine (4Vp) and acrylamide (Ac), which can bind metal ions through the pyridine ring and potentially form H-bonds through the amide group, is used as a model of the paradigm of the enzyme environment where the copper ions are coordinated by a macromolecular ligand possessing controllable functional groups in order to assemble active dicopper(II) center. The high oxidative and hydroxylation activity exhibited by this metallopolymer as well as the ease of structure modification allows for an extensive kinetic investigation of oxygenation /oxidation reactions to reveal the mechanistic similarities and differences from enzymatic and model systems.

4. Applications in detoxification of pollutants

Our civilization, as it is currently operated is not sustainable. It is a consensus among scholars that technology should be practically adjusted to favor sustainability which is the “single most important goal for universities for the next century”. The broader use of alternative oxidants can significantly help the reduction of the environmental burden caused by the metal and chlorine based oxidation processes, historically used in industrial processes. Harnessing the power of green oxidation agents, as in $\text{O}_2$ and $\text{H}_2\text{O}_2$, has gained much attention in the field of catalysis as a mean to alleviate the presence of toxic industrial byproducts in the environment. The current mission of green chemistry is to invent new processes that can successfully replace the existing polluting technologies.

Halogenated aromatic compounds in the form of polychlorinated phenols are used as wood preservatives, pesticides, fungicides, herbicides, insecticides or disinfectants, and are also present in waste of paper mills. They are highly toxic, persistent and regarded as priority pollutants because of their halogen content. Many of these pollutants can be
converted to less dangerous organic products which can eventually be degraded by
different microorganisms. Chlorinated compounds such as pentachlorophenol or 2,4,6
trichlorophenol (2,4,6 TCP) are extremely persistent in the environment because of their
slow degradation by reductive or oxidative enzymatic pathways which lead to overload of
the transformation capacity of microorganisms making efficient chemical treatments
necessary.  

Oxidative degradation is considered as the most advantageous reaction type for this
purpose. Several methods have been reported including bacterial methods and H₂O₂
dependent homogeneous catalysis. Aqueous H₂O₂ is generally regarded as an
environmentally friendly and “green” reagent partly because it gives water as the sole by
product of oxidation. However, H₂O₂ alone does not oxidize chlorinated phenols
effectively without a catalyst, making the design and synthesis of peroxide-using
catalysts a very important component of the decontamination process.

One of the major drawbacks in the use of enzyme systems in catalytic applications is
the low stability and tedious and often low yielding purification process necessary to
obtain the active ingredient which makes their incorporation into large scale
manufacturing processes expensive and impractical. The use of transition metal
complexes as efficient catalysts in oxidative transformations has been extensive, which
serve not only as model systems but also as more accessible alternatives to their enzyme
counterparts. Furthermore, incorporation of these complexes to polymer systems has
gained much attention, since they can be easily prepared in large quantities, can
withstand more extreme conditions and can be potentially recycled to increase the cost
efficiency of catalysis. The polymer matrix can stabilize the catalytic site and furthermore impart selectivity.

5. Reactive oxygen species (ROS), oxidative DNA cleavage and disease

Transition metal ions are essential for a variety of functions in living organisms tuning the activity of a variety of proteins and enzymes. However, metal-ion homeostasis must be well controlled due to the toxicity of abnormal high concentrations of metal ions. Any offset of natural control of their cellular uptake, transport, compartmentalization and binding to the designated tissue or cell constituents can lead to exogenous metals binding to protein ligands other than those designed for this purpose. In a healthy cell, proper mechanisms have been developed for metal ion transport and storage. Metal induced toxicity and carcinogenesis can be related to the ability of some redox-active transition metals like Ni, Co, Cu, Cr and Fe to generate reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals under physiological conditions, enabling oxidative damage of proteins, DNA and other biologically important molecules. Their redox activity can underlay the mechanism of mediation of oxidative damage to cell constituents. Copper-bound ROS, once generated under abnormally high concentrations of metal have been shown to have oxidative activities much higher than free ROS such as hydrogen peroxide. This abnormal ion homeostasis has been related to many neurogenerative disease like Alzheimer’s, Parkinson’s and Creutzfeldt-Jacobs (mad cow) disease. Neurotransmitters such as dopamine, epinephrine, L-dopa and norepinephrine generally known as catecholamines are important in ensuring the proper
transmission of nerve impulses in the brain. Any changes in their concentration can affect the proper function of neurons. Such changes have been reported and related to the oxidative stress observed in the neuropathology of Alzheimer’s disease. Nevertheless, a systematic study of the oxidation mechanism, the degree of oxidation taking place under pathological conditions and the susceptibility of these substrates to the action of the active metal-oxygen intermediates in terms of kinetic constants is lacking.

Furthermore, copper based oxidative “artificial nucleases” have received considerable attention owing to their mechanistic flexibility. However, the exploration of polymer systems as basis for the construction of chemical nucleases has been limited. Understanding the activity and mechanism of these Cu-centered chemical systems can provide information about oxidative damage to nucleic acids and other biomolecules and gain better understanding of mechanism of copper proteins, with possible applications in the fields of aging, cancer research, and oxidative stress-associated apoptosis.

6. Natural Antioxidants as Inhibitors

Several studies have shown that a diet rich in fruits and vegetables is associated with decreased risk of cardiovascular diseases and certain cancers. These beneficial health effects have been attributed in part to the presence of phenolic compounds in dietary plants, which may exert their effects as a result of their antioxidant properties. Flavonoids (Figure 4.7) are a large group of naturally occurring phenolic compounds found in fruit, vegetables, grains, flowers, green and black tea, green tea and red wine. One of the most potent antioxidants is epigallocatechin-3-gallate (EGCG) belonging to the major group of polyphenols found in green tea known as catechins (40% polyphenol content in green tea...
is EGCG. Their beneficial health effects have been associated with their antioxidant properties and have been studied extensively due to their ability to prevent ROS damage by scavenging free radicals and becoming oxidized to result in more stable, less reactive radicals.

The antioxidant activities of flavonoids vary significantly according to their backbone structures and the type of functional groups present in their structures. However, there have been few reports where structure activity relationships have been studied. Their detailed mechanism of action is still under debate, thus a definite relationship between their structural elements, their antioxidant potency, the type of reactive oxygen species they can inhibit, differentiation of their ability to serve as sacrificial substrates or as true inhibitors of oxidative processes, and their potential role in chelation of redox active metal ions must be determined to gain molecular basis about their action.
Figure 4.8. Structures of flavonoids quercetin and catechin and polyphenols gallic acid and ellagic acid
II. Experimental

1. Materials

The buffers MES ((2-[N-Morpholino]ethanesulfonic acid), HEPES (N-[2-hydroxyethyl]piperazine-NN-2-ethanesulfonic acid), TAPS(N-tris[Hydroxylmetyl]methyl–3–aminopropane–sulfonic acid), CAPS(3–(cyclohexylamino)propane sulfonic acid), where purchased from Sigma-Aldrich, the substrates pyrocatechol, THB (1,2,3–trihydroxybenzene), phenol, DTC [di-(t-butyl) catechol], dopamine, L-Dopa, tyrosine, tyramine, epinephrine, norepinephrine, serotonin, deuterated phenol, 2,4-dichlorocatechol, 2,4,6 trichlorocatechol, and 4-chlorocatechol where purchased from Fisher Scientific, the indicator 3-methyl-2-benzothiazolinone hydrazone (MBTH) from Sigma-Aldrich, 30% solution of H$_2$O$_2$ from Fisher, the inhibitors kojic acid, ellagic acid, quercetin, (+)-catechin and the metal salts CuSO$_4$·5H$_2$O, ZnSO$_4$, sodium azide and sodium acetate were purchased from Aldrich. Deionized water of 18MΩ obtained from MiliQ system (Milipore, Bredford, MA) was used to prepare all aqueous solutions. The pQe30Xa DNA plasmid used in all experiments and the kbp markers used to determine the length of the DNA fragments was obtained from Qiagen and agarose for gel preparation and ethidium bromide for gel staining from Aldrich. The copolymer P1 was synthesized according to the procedure described in Chapter 2 section II).
2. Methods

A. Spectrophotometric determination of rates of oxidative reactions

The initial rate of oxidative reactions was monitored using a Varian Cary 50 instrument with variable temperature control. The oxidative reactions of substrates containing catechol or phenol moieties (Figures 4.8-4.9) yields the $o$–quinone moiety. Product formation of the $o$–quinone can be conveniently detected by the formation of an adduct with MBTH, a potent nucleophile which attacks the $o$–quinone at the sixth position thus forming a stable MBTH–quinone adduct with high extinction coefficient $\varepsilon_{500} = 32,500 \text{ m}^{-1}\text{cm}^{-1}$ (Figure 4.10). The reaction rate can be calculated by determining the increase in product absorption as a function of time in the range of 1–5 minutes. It is equal to the slope divided by the extinction coefficient of the MBTH–quinone adduct at the wavelength of maximum absorption $\lambda_{\text{max}}$ of each adduct. The molar absorptivity of $o$-quinone-MBTH adducts may vary, depending on the structure of the substrate. The values of the extinction coefficients used for each product are given in Table 4.3.
Figure 4.9. Structure of substrates containing a phenol moiety used in oxidative reactions.
Figure 4.10. Structure of substrates containing a phenol moiety used in oxidative reactions.
Figure 4.11. Hydroxylation of phenol and oxidation of catechol moieties followed by formation of a quinone–MBTH adduct.
In a typical run, the rates of oxidation of various substrates containing catechol and phenol moieties were determined. Stock solution of P1 (5 mM, based on repeating unit molecular weight of 386.5) is prepared in methanol water and CuSO4 (5 mM) also prepared in water. Few µL of the metal and the polymer P1 are transferred into a cuvette containing buffer MES pH 6.0 (in oxidations) or HEPES pH 8.0 (hydroxylations) and 50% methanol to a final concentration for Cu(II)–P1 of 10 µM or 50 µM (oxidations and hydroxylations respectively). Stock solutions of catechol or phenol and the dye MBTH are prepared in equimolar amounts (0.1 M) and equal amounts are transferred into the cuvette to a final concentration ranging from 0.5 to 10 mM substrate and dye. A stock solution of 9.8 M of H2O2 was used and diluted to a final concentrations ranging from 1–100 mM in the reaction mixture. In the absence of hydrogen peroxide the amount of oxygen available for reaction is only 0.27 mM for 100% air saturated water. Typical changes in the spectrum for phenol and catechol oxidation are shown in Figure 4.11. The time-dependent changes are plotted in the insets, where the slopes give the initial rates.
Figure 4.12. (1) Hydroxylation of 1.0 mM phenol by 0.5 mM Cu(II)–P1 at pH 8.0 in air (2) Oxidation of 4.0 mM catechol by 10 μM Cu(II)–P1 at pH 6.0 in 8 mM H₂O₂
<table>
<thead>
<tr>
<th>Substrate</th>
<th>ε (M⁻¹ cm⁻¹) MBTH-Quinone</th>
<th>λ max (nm)</th>
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</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>32,500⁹⁵</td>
<td>500</td>
</tr>
<tr>
<td>THB</td>
<td>32,500</td>
<td>500</td>
</tr>
<tr>
<td>DTC</td>
<td>1,910 (without MBTH)</td>
<td>420</td>
</tr>
<tr>
<td>4, 5-dichlorocatechol</td>
<td>32,500</td>
<td>500</td>
</tr>
<tr>
<td>Dopamine</td>
<td>42,500⁹⁶</td>
<td>500</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>32,500</td>
<td>500</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>32,500</td>
<td>500</td>
</tr>
<tr>
<td>Serotonin</td>
<td>14,000⁹⁸</td>
<td>500</td>
</tr>
<tr>
<td>L-dopa</td>
<td>38,000⁹⁶</td>
<td>500</td>
</tr>
<tr>
<td>Phenol</td>
<td>32,500⁹⁵</td>
<td>500</td>
</tr>
<tr>
<td>4-chlorophenol</td>
<td>24,900⁹⁸</td>
<td>500</td>
</tr>
<tr>
<td>2,4-dichlorophenol</td>
<td>3,200⁹⁷</td>
<td>500</td>
</tr>
<tr>
<td>2,4,6-trichlorophenol</td>
<td>5,700⁹⁷</td>
<td>500</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>48,000⁹⁵</td>
<td>500</td>
</tr>
<tr>
<td>Tyramine</td>
<td>42,500⁹⁵</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 4.4. Extinction coefficients for the oxidation products of various substrates used in the oxidation studies catalyzed by Cu(II)-P₁
B. DNA cleavage assays

The activity of Cu$^{2+}$-P1 toward DNA cleavage was determined by incubating 50.0 µM of the complex in terms of [Cu$^{2+}$] with 0.15 µg pQE30Xa plasmid in the presence of 1.5% (0.49 M) H$_2$O$_2$ and 0.1 M HEPES pH 8.0 in a volume of 12.0 µL for several different time periods. The oxidative cleavage of plasmid DNA was observed by means of 1% agarose gel electrophoresis (150 V, 1× Tris-acetate buffer for one hour). All DNA cleavage assays were buffered with 50-mM HEPES at pH 8.0. The experiment was repeated three times to ensure consistency. All plastic ware was demetallized with EDTA and rinsed with 18 MΩ water.
3. Pre-equilibrium kinetics and catalytic constants

A catalytic reaction may follow a two step process analogous to enzyme catalysis comprised of substrate (S) binding to the catalyst leading to the formation of the ES complex which in turn leads to the product (P) formation. In this reaction scheme, it is assumed that the substrate binding reaction is at equilibrium (Eq. 1).

\[
\begin{align*}
E + S & \rightleftharpoons_{k_1}^{k_{-1}} ES \\
& \stackrel{k_{\text{cat}}}{\longrightarrow} E + P
\end{align*}
\]

Eq. 1

The rate of an enzyme catalyzed reaction is limited by the rate of breakdown of the ES complex and can therefore be expressed as in Eq. (2)

\[
V = k_{\text{cat}} [ES]
\]

Eq. 2

The above rate expression describes the pseudo first-order reaction obtained in excess amount of substrate S. In order to determine the rate of the reaction, the steady state approximation is used according to which the concentration of E-S complex remains nearly constant over a prolonged period of time thus achieving equilibrium shortly after the beginning of the reaction and the rate of its production \((k_1[E][S])\) is equal to the rate
of its decomposition \((k_{\text{cat}} + k_{-1}[\text{ES}])\). In the case of metallopolymer catalysis, by equating the two rates, the dissociation of the substrates can be given by Eq. 3.

\[
\begin{align*}
\text{Cu}^{II} - \text{P1} + \text{S} & \xrightleftharpoons[k_{-1}]{k_1} \text{P1-Cu}^{II} - \text{S} & \xrightarrow[k_{\text{cat}}]{\text{Cu}^{II} - \text{P1} + \text{Prod.}} \\
\end{align*}
\]

Eq. 3

\[
\begin{align*}
\text{rate} &= \frac{k_{\text{cat}}[\text{Cu}^{II} - \text{P1}][\text{S}]}{K' + [\text{S}]}
\end{align*}
\]

Eq. 4

The catalysis by Cu-P1 can be described as the binding of the substrate to the catalytic metal center to form an intermediate \(\text{P1-Cu}^{II} - \text{S}\) analogous to the ES complex in enzyme catalysis followed by the conversion of the bound substrate into products (Eq. (4)). The rate law for this reaction mechanism is thus expressed as in Eq. (5) analogous to the Michaelis-Menten equation with \(K' = (k_{-1} + k_{\text{cat}})/k_1\) as the apparent dissociation constant of the \(\text{P1-Cu}^{II} - \text{S}\) complex, assuming that the concentration of the complex is much lower than that of unbound substrate S. Herein, the initial rates for the oxidation of several catechol-type of substrates S are determined at different concentrations of S and fitted to Eq. (4) to yield the first order rate constant \(k_{\text{cat}}\) (Eq.(2)) and the substrate dissociation constants \(K' = (k_{\text{cat}} + k_{-1})/k_1\).

The oxidation of catechol type of substrates in presence of Cu(II)-P1 metallopolymer complex follows kinetic behavior similar to enzymes as described above yielding a non
linear plot when the initial rate of the oxidative reactions is plotted against substrate concentration in excess of the catalyst Cu-P1 showing saturation behavior (Eq. 5)

\[ K' = \frac{\left( k_{\text{cat}} + k_{-1} \right)}{k_1}. \]  

Eq. 5

4. Bisubstrate reaction mechanism

In enzymatic systems it is common that more than one substrates can interact with the active site. In ordered sequential mechanisms, substrates interact with the enzyme and products are released in a specific order while in a random mechanism the binding of the two substrates does not take place in a specified order. In a random bisubstrate mechanisms each of the substrates can interact with free enzyme as well as with the enzyme substrate complex. In some cases, the binding of one substrate can affect the dissociation constant of the other. We consider \( K_{iA} \) and \( K_{iB} \) the true dissociation constants of substrates A and B, respectively, in the absence of the second substrate, and \( K_A \) and \( K_B \) the apparent dissociation constants of each substrate in presence of saturating amounts of the other. The reaction can be described as in the scheme below (Fig. 4.12).
Figure 4.13. Rapid equilibrium binding scheme for a random bisubstrate reaction

For a random bi-substrate reaction, the rate of catalysis of one substrate A is systematically determined at various concentrations of the other substrate B (i.e., rate vs. [A] at various [B]) and the data analyzed by the use of the Hanes plots (Eq. 6) by plotting [S]/rate as a function of [S], wherein S can be fixed to either A or B and B is the other variable once A is fixed. The secondary plots of the slope \((1 + K'_B/[B])/V_{\text{max}}\) and the y-intercept \((K'_A/V_{\text{max}})(1 + K'_{iA} K'_B/[B]K'_A)\) from the Hanes plot (Eq. 6 and Figure 4.13) yield the dissociation constants.

\[
\frac{[A]}{V_0} = \frac{1 + K_B/[B]}{V_{\text{max}}} [A] + \frac{K_A}{V_{\text{max}}} \left(1 + \frac{K_{iA} K_B}{[B]K_A}\right) \quad \text{Eq. (6)}
\]
Figure 4.14. Hanes plot of one substrate as a function of the other.
5. Inhibition studies

An inhibitor is a compound that decreases the rate of a catalyzed reaction. Inhibition can be reversible or irreversible depending on the nature of interactions with the active site. Reversible inhibition can be competitive, uncompetitive, or linear mixed type, each affecting $K_m$ and $V_{max}$ in a specific fashion.

a) Competitive Inhibition

In this type of reversible inhibition, an inhibitor competes with the substrate for binding to the active site. The result of this competition is the increase of the substrate’s dissociation constant $K_s$ in the presence of the inhibitor while the maximal rate is unaffected.

The equation that gives the rate of the reaction in the presence of a competitive inhibitor is given in Eq.7 The $K_i$ is the dissociation constant of inhibitor I.
b) Linear Mixed type Inhibition

In this type of reversible inhibition, a compound can interact with both the free catalyst and the catalyst- substrate complex at a site other than the active site. The result of this type of inhibition is the decrease of the maximal rate $V_{\text{max}}$ and an increase in the substrate dissociation constant. The rate of the reaction is given by Eq. 8.

$$V_0 = \frac{V_{\text{max}}[S]}{1 + \frac{[I]}{K_i}K_m + [S]}$$  \hspace{1cm} \text{Eq. 7}$$

$$V_0 = \frac{V_{\text{max}}[S]}{1 + \frac{[I]}{K_i}K_m + \left(1 + \frac{[I]}{aK_i}\right)[S]}$$  \hspace{1cm} \text{Eq. 8}$$
III. Results and Discussion

1. Polyphenol Oxidation

The catalytic activity of Cu$^{II}$-P1 was investigated toward the oxidation of the polyphenol 1,2,3-trihydroxybenzene (THB) in the presence of increasing amounts of H$_2$O$_2$ (Fig. 4.14a). The reaction reaches a plateau at high [THB], suggesting a pre-equilibrium kinetic pathway. The reaction in the presence of 0.27% (or 80 mM) H$_2$O$_2$ exhibits first-order rate constant (Eq. 3) $k_{cat} = 0.152$ s$^{-1}$ with an apparent dissociation constant $K'_{THB} = 2.40$ mM and a second-order catalytic efficiency $k_{cat}/K'_{THB} = 63.3$ M$^{-1}$ s$^{-1}$. The oxidation of 8.0 mM THB by Cu$^{II}$-P1 as a function of [H$_2$O$_2$] is also not linear and can be fitted to Eq. (4) to give $k_{cat} = 0.241$ s$^{-1}$, $K'_{H_2O_2} = 9.70$ mM, and $k_{cat}/K'_{H_2O_2} = 24.8$ M$^{-1}$ s$^{-1}$ (Fig. 4.15a).

For comparison, the background oxidation of THB was determined in the absence of Cu$^{II}$-P1 and found to have rate constant $k_o = 5.38 \times 10^{-6}$ s$^{-1}$ under the pseudo-first-order conditions with $rate = k_o [THB]$ at 80mM of H$_2$O$_2$ and $k_o = 3.45 \times 10^{-6}$ s$^{-1}$ for $rate = k_o$ [H$_2$O$_2$] at THB = 8 mM. The rate constants of the catalyzed reaction was compared with the background oxidation of THB and the catalytic proficiency for THB oxidation is determined to be $k_{cat}/k_o = 2.83 \times 10^4$ using the THB saturation plot at highest peroxide of 80 mM (Figure 4.14a) and comparing to background rate at 80 mM peroxide (see Fig. 4.15). Similarly, the catalytic proficiency is $k_{cat}/k_o = 3.38 \times 10^4$ using Figure 4.15a and the background oxidation rate at [THB] = 8 mM. In comparison, the oxidation of THB by the redox-inactive Zn$^{II}$-P1 complex was negligible under similar conditions.
Figure 4.15. (a) Initial rate of THB oxidation by Cu$^{II}$-P1 in the presence of 0, 1.0, 2.0, 5.0, 40, and 80 mM H$_2$O$_2$ (from bottom), and fitting of the data to Eq. 2. (b) Hanes plot of the data from (a) from which the dissociation constants in Eq. 3 can be obtained.
Figure 4.16. (a) Initial rate of THB oxidation by Cu$^{II}$-P1 in the presence of 0.4, 0.8, 2.0, 4.0, mM THB (from bottom) varying the [H$_2$O$_2$] (b) Hanes plot of the data from (a)
Figure 4.17. Comparison of oxidation of THB in presence of 10 µM Cu^{II} and of Cu^{II}-P1 in 80 mM H_{2}O_{2} at pH 6.0 MES buffer in 50% methanol. The solid lines are the linear fitting the hyperbolic fitting of the data.
A noticeable rate of THB oxidation was still detected without H₂O₂ and it was significantly higher than its auto-oxidation rate (Fig. 4.14a). In the absence of H₂O₂ molecular oxygen is the oxidizing agent at a concentration of only 0.27 mM at 25 °C for 100% air saturated water. Aerobic oxidation of THB by Cu²⁺-P₁ as a function of [THB] without H₂O₂ follows pre-equilibrium kinetics (Eq. 3-4), giving rate constants \( k_{\text{cat}} = 0.012 \text{ s}^{-1} \), \( K' = 1.02 \text{ mM} \), and \( k_{\text{cat}}/K' = 11.8 \text{ M}^{-1} \text{ s}^{-1} \) (●, Fig. 4a). The first-order catalytic proficiency \( k_{\text{cat}}/k_o \) in this case is \( 3.12 \times 10^4 \) folds \( (k_o = 3.85 \times 10^{-7} \text{ s}^{-1} \) under the same conditions).

A direct comparison of the oxidation rates for THB catalyzed by Cu²⁺ ion and Cu²⁺-P₁ is shown in Figure 4.16. The oxidation rates in the presence of molecular oxygen are higher for the Cu²⁺-P₁ complex when compared with the rates of Cu²⁺ in the absence of polymer. Moreover, the rate as function of [THB] is not linear showing saturation behavior which is similar to the response expected for oxidative enzymes when the rate is determined in presence of increasing substrate concentrations.

Similarly, the commonly used catechol substrate 3,5-di-\( t \)-butylcatechol (DTC) was effectively oxidized by Cu²⁺-P₁ with 50 mM H₂O₂ and follow the same kinetics as THB to give \( k_{\text{cat}} = 1.55 \text{ s}^{-1} \), \( K_{\text{DTC}}' = 2.45 \text{ mM} \), and \( k_{\text{cat}}/K_{\text{DTC}}' = 633 \text{ M}^{-1} \text{ s}^{-1} \) (○, Figure 4.17) affording a significant first-order catalytic proficiency of 0.267 million with respect to the auto-oxidation rate constant \( k_o \) of \( 5.80 \times 10^{-6} \text{ s}^{-1} \) under the same conditions (10mM DTC and 50 mM H₂O₂). Oxidation of DTC as a function of [H₂O₂] at 10 mM DTC also follows a pre-equilibrium kinetics, yielding \( k_{\text{cat}} = 0.65 \text{ s}^{-1} \) and \( K'_{\text{H₂O₂}} = 6.58 \text{ mM} \) (Figure 4.17). Aerobic oxidation of DTC by Cu²⁺-P₁ at pH 6.0 without H₂O₂ (●, Figure 4.18) yields \( k_{\text{cat}} = 0.065 \text{ s}^{-1} \),
Figure 4.18. Rate of DTC oxidation as a function of $[\text{H}_2\text{O}_2]$ at pH 6.0 MES 0.1 M in 50% methanol using 10 µM Cu(II)-P1.
Figure 4.19. Oxidation of DTC in air (●) and presence (○) of 50 mM H$_2$O$_2$ in 10 μM Cu$^{II}$-P1 and Hanes analysis of the profile (bottom).
$K' = 0.16 \text{ mM, and } k_{\text{cat}}/K' = 406 \text{ M}^{-1} \text{ s}^{-1}$, which affords $k_{\text{cat}}/k_0$ of $9.60 \times 10^4$ folds (with $k_0$ determined to be $6.77 \times 10^{-7} \text{ s}^{-1}$ under the same conditions).

The oxidation constants for the prototypical substrate catechol were also determined in presence of molecular oxygen and in presence of varying amounts of $\text{H}_2\text{O}_2$ and were compared to the parameters obtained for THB and DTC. The results indicate that this substrate exhibits similar behavior showing saturation at high concentrations of substrate and at high concentration of $\text{H}_2\text{O}_2$ (Figures 4.18-4.19). Using the same analysis as in the case of DTC and THB, the catalytic parameters for catechol were determined to be $k_{\text{cat}} = 0.172 \text{ s}^{-1}$, $K' = 0.90 \text{ mM}$, and $k_{\text{cat}}/K' = 191 \text{ M}^{-1} \text{ s}^{-1}$ at saturating amount of hydrogen peroxide (Figure 4.18) and in air (●, Figure 4.19) the kinetic parameters are $k_{\text{cat}} = 1.2 \times 10^{-4} \text{ s}^{-1}$, $K' = 0.6 \text{ mM}$, and $k_{\text{cat}}/K' = 195 \text{ M}^{-1} \text{ s}^{-1}$.

The rate constants observed here for the three catechol type of substrates are comparable or higher than those of a number of Cu$^{\text{II}}$ complexes and Fe$^{\text{III}}$-porphyrin systems$^{101}$ toward the oxidation of catechol and derivatives (Table 4.2), and are only ~150–350 times lower than those of horseradish peroxidase toward THB oxidation.$^{102}$
Figure 4.20. Saturation of H$_2$O$_2$ in oxidation of 4 mM catechol by 10 mM Cu$^{II}$-P$_1$ in 50% methanol-buffer 0.1 M MES pH 6.0.
Figure 4.21. Catechol saturation profile in various amounts of \( \text{H}_2\text{O}_2 \) (top) and subsequent Hanes Plot analysis of the data (bottom).
Since all the substrates used and H$_2$O$_2$ can potentially bind to the catalyst Cu(II)-P$_1$ as concluded by the saturation behavior of the rate as function of substrate or H$_2$O$_2$ at high substrate concentrations, the data are analyzed with random bi-substrate mechanism by the use of the Hanes plot in which the ratio of substrate concentration over rate of oxidation is plotted against the concentration of substrate at different amounts of H$_2$O$_2$, where the slope is \( (1 + K'_{H_2O_2}/[H_2O_2])/V_{max} \) and the y-intercept is \( 1 + (K'_{iTHB} K'_{H_2O_2})/([H_2O_2]K'_{THB}) \) as seen in Eq. 7.

\[
\frac{[THB]}{V_0} = \left( \frac{1 + K'_{H_2O_2}/[H_2O_2]}{V_{max}} \right)[THB] + \frac{K'_{THB}}{V_{max}} \left( 1 + \frac{K'_{iTHB} K'_{H_2O_2}}{[H_2O_2]K'_{THB}} \right)
\]

Eq. 7

The secondary plots of the slopes and the y-intercepts from Figures 4.14, 4.15 and 4.18, 4.20 for THB, DTC and catechol respectively versus 1/[H$_2$O$_2$] according to the Hanes analysis for a random biusubstate system afford the dissociation constants. The \( K'_{THB}/K'_{iTHB} \) ratio is 6.6/1.8 = 3.7, the \( K'_{DTC}/K'_{iDTC} \) ratio is 2.56 / 0.109 = 25 and the \( K'_{catech}/K'_{i catech} \) is 2.5/0.3 = 8.3, all consistently larger than 1.0, which indicates the binding of H$_2$O$_2$ to the active center significantly decreases the binding affinity for all these substrates.\(^9\) Where possible, plotting of the oxidation rate as function of [H$_2$O$_2$] at increasing concentrations of [S] as shown in Fig. 4.15 for THB, which is analogous to Fig.4.14, enables the calculation of the intrinsic and apparent dissociation constants for the oxidation substrates and H$_2$O$_2$ making their comparison possible. From Fig. 4.15 the
intrinsic dissociation constant for H$_2$O$_2$ K'$_{H2O2}$ can be obtained, giving a K'$_{H2O2}$ / K'$_{H2O2}$ ratio of 9.5 which is larger than one. The Hanes analysis results suggest that the binding of H$_2$O$_2$ influences the binding of the oxidation substrates and vice versa as reflected by the increase in dissociation constants of all the oxidation substrates and of H$_2$O$_2$ relative to the intrinsic dissociation constants.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{\text{cat}}(\text{s}^{-1})$</th>
<th>$K_m$ mM</th>
<th>$k_o(\text{s}^{-1})$</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{\text{rel}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>THB</td>
<td>$(4.4\pm0.32) \times 10^{-3}$</td>
<td>0.1±0.015</td>
<td>$1.1 \times 10^{-6}$</td>
<td>44</td>
<td>4,000</td>
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<tr>
<td>Catechol</td>
<td>$(2.7\pm0.4) \times 10^{-2}$</td>
<td>2.8±0.52</td>
<td>$7.2 \times 10^{-6}$</td>
<td>9.6</td>
<td>3,750</td>
</tr>
<tr>
<td>DTC</td>
<td>$(5.5\pm0.7) \times 10^{-2}$</td>
<td>0.12±0.03</td>
<td>$4.2 \times 10^{-6}$</td>
<td>458</td>
<td>13,095</td>
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<table>
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<th>Substrate</th>
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<th>$K_m$ mM</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_o$ (s$^{-1}$)</th>
<th>$k_{\text{rel}}$</th>
</tr>
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<tr>
<td>THB</td>
<td>0.241±0.01</td>
<td>4.0±0.5</td>
<td>60</td>
<td>$1.1 \times 10^{-5}$</td>
<td>21,900</td>
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<tr>
<td>Catechol</td>
<td>0.111±0.001</td>
<td>0.9±0.1</td>
<td>123</td>
<td>$7.2 \times 10^{-6}$</td>
<td>15,417</td>
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<tr>
<td>DTC</td>
<td>0.164±0.04</td>
<td>2.4±0.48</td>
<td>68</td>
<td>$4.2 \times 10^{-6}$</td>
<td>39,000</td>
</tr>
</tbody>
</table>

**Table 4.5.** Kinetic parameters for oxidation of various substrates in air (top) and in presence of saturating amounts of H$_2$O$_2$ (bottom).
Substrate (A) & $K_{ia}$ (intrinsic) mM & $K_A$ (apparent) mM & $K_{H2O2}$ (apparent) mM \\
--- & --- & --- & --- \\
Catechol & 0.3 & 2.5 & 27.0 \\
THB & 1.8 & 6.6 & 9.5 \\
DTC & 0.10 & 1.0 & 2.6 \\

**Table 4.6.** Calculated apparent and intrinsic dissociation constants for catechol, THB and DTC
2. Metal interactions in the dicopper center and its role in oxidative reactions

The oxidation reaction in the presence of polymer solution was not enhanced when compared to the self-oxidation of various substrates containing the catechol moiety. The reaction was significantly enhanced only when both Cu(II) and P1 were present in the reaction medium. Furthermore, the oxidation rate was considerably higher in the presence of micromolar amounts of Cu(II)-P1 when compared to the rate in presence of equivalent amounts of Cu(II) but in the absence of polymer (Figure 4. 21).

To further explore the possible interactions or cooperativity of the metal centers, CuII was added to P1 in the presence of the redox-inactive ZnII at a constant [CuII] + [ZnII]. Gradual replacement of Cu2+ in Cu-P1 with redox-inactive Zn2+ is expected to serve as a practical method for addressing the nature of the active center, such as cooperativity, by steady inactivation of the catalyst through the dilution with Zn2+. A linear correlation between the activity and the mole fraction of CuII should be observed for simple mononuclear catalysis if there is no cooperativity among CuII active sites. On the contrary, a sigmoidal activity profile was observed toward the oxidation of THB with and without 15 mM H2O2, which can be fitted to the Hill equation (Eq. 8) giving Hill coefficients \( \theta \) of 1.4 and 2.8, respectively (Fig. 4.22). A sigmoidal response was also observed when catechol was used as a substrate for the same experiment (Fig.4.23), and the fitting to Hill equation gave a coefficient \( \theta \) equal to 2.5 comparable to the coefficient for THB. This observation suggests a possible presence of cooperativity among metal centers for polyphenol oxidation by CuII-P1.
\[
\frac{V_o}{V_{max}} = \frac{[\text{Cu-P1}^9]}{K^6 + [\text{Cu-P1}^6]} \quad (\text{Eq. 8})
\]

**Figure 4.22.** Dose dependence of catechol oxidation (0.1 mM) by Cu–P1 (●) compared to equivalent amounts of Cu²⁺ (▽) in 50% methanol–buffer 0.1 M MES pH 6.0
**Figure 4.23.** Oxidative activity of Cu-P1 toward THB in the absence (●) and presence (○) of 15 mM H$_2$O$_2$ as a function of Cu$^{II}$ mole fraction at a fixed total concentration of Cu$^{II}$ and Zn$^{II}$. The traces are fittings to the Hill equation. All the experiments were performed in 1:1 methanol/25mM MES buffer at pH 6.0 and 25 °C.
**Figure 4.24.** Oxidative activity of Cu-P1 toward catechol in air as a function of Cu$^{II}$ mole fraction at a fixed total concentration of Cu$^{II}$ and Zn$^{II}$. The trace is a fittings to the Hill equation. The experiment was performed in 1:1 methanol/25mM MES buffer at pH 6.0 and 25 °C.
3. Influence of azide in oxidative reactions

Coordination of azide to copper centers can adopt three main modes, the $\mu$-1,3 coordination, a terminal coordination or in a $\mu$-1,1 coordination mode (Figure 4.25). It is known from previous studies$^{100-101}$ that azide is capable of forming complexes with many copper enzymes, and it can act as an inhibitor. Moreover, in a study using dinuclear copper(II) complexes it has been shown that more than one azide molecules can bind to the dicopper center leading to progressive lowering of the electronic communication between the metal centers.$^{102}$

Azide’s toxicity to copper enzymes is attributed to its ability to coordinate to the active metal center changing its coordination number and conformation preventing the formation of a key intermediate responsible for the activity of the enzyme or by blocking the binding of molecular oxygen. Reports of azide acting as an activator are not common, but it has been reported that azide can act as an activator in polyphenol oxidase (PPO) from tobacco. In this report, the activity increases at low molar ratio of azide to PPO II up to 1.0 equivalents of azide and it decreases until 2.0 equivalents and at higher equivalents it becomes inhibitory suggesting that azide can act both as inhibitor and activator depending on the concentration of azide present.$^{103}$
Figure 4.25. Binding modes of Azide to dicopper centers of proteins and model complexes.

Azide had an activation effect in the THB and catechol oxidation by Cu(II)-P1 (Fig.4.26) when up to 1 mM azide is added in the reaction medium and after that concentration is exceeded, the activity gradually decreases until it reaches an activity equal to what is observed in the absence of azide. Optical titration of the catalyst Cu(II)-P1 with azide in methanol produces a peak at 410 nm (Fig. 4. 27) with intensity that increases when the concentration of azide increases reaching saturation at 1 mM azide, and the dissociation constant for azide is calculated to be $K'_{\text{azide}} = 1.1$ mM. Furthermore, the Cu(II) titration of P1 in the presence of equivalent azide and of the redox inactive Zn$^{II}$ performed at a constant [Cu$^{II}$]+[Zn$^{II}$] while monitoring the THB oxidation is not linear and is markedly different when compared with the titration in the absence of azide, in the presence and absence of H$_2$O$_2$ which suggest the possible synergy of two copper
ions in the formation of the active intermediate (Fig. 4.28). Taken together, the results suggest that azide binds to the copper center and the possible binding mode is terminal based on the similarity of the optical spectra and the activation pattern observed in the azide bound-PPO II.\textsuperscript{104}
Figure 4.26. Azide influence in oxidation of 5 mM catechol (top) and 5 mM THB (bottom) in 10 μM Cu(II)–P1 in air at pH 6.0 MES buffer in 50% methanol
Figure 4.27. (Top) Azide titration into 0.1 mM Cu(II)–P1 in methanol–buffer 0.1 M MES pH 6.0 (bottom) Increase in the absorption at 410 nm as a function of [azide].
Figure 4.28. Cu–Zn dilution comparison experiments in presence (○) and absence (●) of 2mM Azide (a) in air (b) in presence of 15 mM H$_2$O$_2$ using 4mM THB as the oxidation substrate and 20 µM Cu(II)–P1 at pH 6.0 MES 0.1 M in 50% methanol
4. Oxidation of neurotransmitters

Neurotransmitters are small molecules that function by facilitating the transimition of signals to neurons. Depletion of their concentration has been connected to some of the symptoms observed in neurogenerative diseases. It has been shown that metal centered ROS can cause the oxidation of phenol and catechol type of substrates quite efficiently. Dopamine, epinephrine, norepinephrine and L-dopa are all involved in transimission of neural signals, and also have a catechol moiety in their structure and as a result they are susceptible to the action of active oxidative species. The oxidative activity of Cu(II)-P1 towards polyphenols was shown to be quite efficient therefore this complex can be used as a model to study the effect of metal center oxidations on the levels of neurotransmitters, which can give a clue about the extend of the potential damage caused by oxidative stress related pathways on the normal concentration of these molecules.

The oxidative activity of Cu(II)-P1 towards several neurotransmitters was determined at pH 6.0 MES buffer in 50% methanol, in air or in presence of H2O2. The reaction seems to follow similar mechanism as the oxidation of the polyphenols showing saturation both at high concentrations of neurotransmitters, when [H2O2] is kept constant and at high concentration of H2O2 when the neurotransmitter concentration remains constant (Fig. 4.29-4.32). Fitting of the data to Eq. 4 (section 4.3.4) assuming a pre-equilibrium kinetic pathway, gives the catalytic turnover $k_{cat}$ and the dissociation constant $K_{m}$ for each substrate in air and in presence of saturating amounts of H2O2 (Tables 4.7–4.8). Comparing the catalytic turnover of each substrate with the oxidation rate constant under the same experimental conditions but in the absence of catalyst Cu(II)–P1 gives the rate
acceleration due to Cu(II)-P1 ($K_{rel}$). A list of all kinetic parameters and the comparison to background rates in air and in saturating amounts of H$_2$O$_2$ are given in Tables 4.7 and 4.8.

**Figure 4.29.** (Top) Saturation profiles of norepinephrine oxidation in (0, ●; 20, ▼; 40, ■; and 100, □, mM H$_2$O$_2$ in presence of 20 µM Cu(II)–P1 in methanol–buffer pH 6.0 MES 0.1M (Bottom) Hanes analysis plot from the top figure.
Figure 4.30. (Top) Saturation profiles of dopamine oxidation in various [H₂O₂] in presence of 20 µM Cu(II)–P1 in methanol–buffer pH 6.0 MES 0.1M (bottom) Hanes analysis plot.
Figure 4.31. Saturation of peroxide in oxidation of 4 mM dopamine by 20 µM Cu(II)–P1 at pH 6.0 MES 0.1 M in 50% methanol.
Figure 4.32. (Top) Saturation profiles of epinephrine oxidation in various [H2O2] in the presence of 20 µM Cu(II)–P1 in methanol–buffer pH 6.0 MES 0.1M (Bottom) Hanes analysis plot.
Figure 4.33. Effect of H$_2$O$_2$ in the oxidation of 10 mM L–Dopa by 10 µM Cu(II)–P1 at pH 6.0 MES buffer in 50% methanol.
It is worth noting that the chirality of the substrate had no effect on the oxidation rates as determined by measuring the oxidation rates of (+) and (-) epinephrine and norepinephrine (rates of oxidation of 10 mM (-)epinephrine or (-)norepinephrine in 100mM H$_2$O$_2$ are the identical to the rates of oxidation of (+)epinephrine and (+) norepinephrine).

Furthermore, the rate enhancement observed for all the neurotransmitters were comparable with the rate accelerators observed for THB and catechol (Table 4.5 section 4.3.1). The only substrate showing ~100 times smaller rate acceleration among neurotransmitters is DOPA. This is the only substrate possessing a carboxyl group which is fully deprotonated under the reaction conditions and is most likely the reason for the least favorable interaction with Cu(II)-P1 in this reaction.

Since all neurotransmitters and H$_2$O$_2$ exhibit saturation behavior, the data in Fig. 4.29-4.32 are analyzed with a random bi-substrate mechanism by the use of the Hanes plot in which the ratio of substrate concentration over rate of oxidation is plotted against the substrate concentration at increasing amounts of H$_2$O$_2$ (section 4.2.4). The analysis the intrinsic and the apparent dissociation constants of each substrate and of H$_2$O$_2$, which allows comparison of the effect of the substrate binding to binding of H$_2$O$_2$ and vice versa. The results are summarized in Table 4.9. The dissociation constants in presence and absence of H$_2$O$_2$ (K$_{app}$/K$_i$ ) for dopamine give a ratio of 600, for epinephrine the ratio of the dissociation constants is 3.4 and for norepinephrine is 2.5 all greater than unity which suggests that H$_2$O$_2$ binding has an effect on the binding of each of these substrates, suggesting that peroxide and substrate bind in close proximity which support their interaction with the metal center during the reaction.
Table 4.7. Kinetic parameters for oxidation of various neurotransmitters in air without H$_2$O$_2$
Table 4.8. Kinetic parameters for oxidation of various neurotransmitters in presence of saturating amounts of H₂O₂

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{\text{cat}}$ (s⁻¹)</th>
<th>Kₘ (mM)</th>
<th>$k_{\text{cat}} / Kₘ$ (M⁻¹s⁻¹)</th>
<th>$k_o$ (s⁻¹)</th>
<th>Kₐrel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>0.107±0.02</td>
<td>0.2±0.019</td>
<td>535</td>
<td>3.2×10⁻⁶</td>
<td>33,430</td>
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<tr>
<td>(−) Epinephrine</td>
<td>0.133±0.007</td>
<td>3.7±0.92</td>
<td>36</td>
<td>4.7×10⁻⁶</td>
<td>28,290</td>
</tr>
<tr>
<td>(−) Norepinephrine</td>
<td>0.022±0.0015</td>
<td>2.5±0.62</td>
<td>9.0</td>
<td>4.1×10⁻⁶</td>
<td>5,366</td>
</tr>
<tr>
<td>L–dopa</td>
<td>3.7×10⁻³</td>
<td>–</td>
<td>–</td>
<td>8.9×10⁻⁶</td>
<td>416</td>
</tr>
</tbody>
</table>
Table 4.9. Calculated apparent and intrinsic dissociation constants for dopamine, epinephrine and norepinephrine

<table>
<thead>
<tr>
<th>Substrate (A)</th>
<th>$K_{iA}$ (intrinsic) mM</th>
<th>$K_A$ (apparent) mM</th>
<th>$K_{H2O2}$ (apparent) mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>$5.0 \times 10^{-5}$</td>
<td>0.03</td>
<td>7.0</td>
</tr>
<tr>
<td>(-)Epinephrine</td>
<td>4.4</td>
<td>13.7</td>
<td>2.6</td>
</tr>
<tr>
<td>(-)Norepinephrine</td>
<td>0.7</td>
<td>1.7</td>
<td>4.1</td>
</tr>
</tbody>
</table>
5. Inhibition of polyphenol oxidation

Kojic acid is a commonly used inhibitor of catechol oxidases and tyrosinases due to its similarity with catechol structure, thus serving as a competitive inhibitor of catechol toward oxidation reactions of polyphenol oxidases in general. The inhibition of kojic acid in the oxidation of THB catalyzed by 10 µM Cu(II)-P1 was performed in the presence of a saturating amount of peroxide while varying the THB concentration and vice versa. The rationale of this experiment is to reveal the differences in the binding sites of these two molecules which were shown to interact with the catalytic metal center in Cu(II)-P1.

The inhibition observed for THB seems to be close to a competitive pattern (Figures 4.36-4.37), with an inhibition constant was determined to be $K_{iTHB} = 0.2$ mM while the inhibition of kojic acid towards peroxide binding is mixed type close to a non-competitive pattern with inhibition constants of $K_{iH2O2} = 4.2$ mM and $aK_{iH2O2} = 5.9$ mM. Since kojic acid is a substrate analog, the competitive inhibition pattern suggest it binds to the same site as the substrate THB and catechol, which provides further support for the similarity of the oxidative mechanism with the mechanism of catechol oxidases. The mixed type inhibition toward H$_2$O$_2$ suggests that the inhibitor and H$_2$O$_2$ binding is not mutually exclusive and involves different binding sites, which also implies that the substrate and H$_2$O$_2$ have different binding sites on the metal center.
**Figure 4.34.** Effect of kojic acid in oxidation of 4mM THB by 10µM Cu(II)–**P1** in methanol–buffer 0.1 M MES with 50% methanol in air. The structure of the kojic acid inhibitor is given in the inset.
Figure 4.35. Inhibition pattern of kojic acid in oxidation of THB at saturating amounts of H$_2$O$_2$ (0.1M) catalyzed by 10µM Cu(II)–P1 in methanol–buffer 0.1 M MES.
Figure 4.36. Effect of kojic acid in saturation profile of $\text{H}_2\text{O}_2$ at saturating amounts of THB (2mM) and 10$\mu$M Cu(II)--P1 in methanol--buffer 0.1 M MES.
Figure 4.37. Inhibition pattern of kojic acid on the peroxide saturation, from data shown in Fig. 4.36.
6. Oxidative Plasmid DNA Cleavage

Cu\textsuperscript{II}-P1 shows a significant activity toward oxidative cleavage of plasmid DNA (Fig. 4.37), wherein nicking of the supercoiled (sc) plasmid by 50.0 µM RU of Cu\textsuperscript{II}-P1 rapidly occurs within 10 min (lane 1) to yield nicked circular (nc) and linear (ln) (which is consistent with ~3.5 kbp of the plasmid pQE30Xa) forms of the plasmid. The nc form is then slowly cleaved into ln form and still retains a significant amount after two hours (lane 6). The nc form shows a first-order decay with a rate constant $k_1 = 0.0095$ min\(^{-1}\) (●, Fig. 4.37), suggesting possible double-stranded (ds) cleavage of the nc form to yield the ln form in a single step. However, the formation of the ln form follows a bi-exponential kinetics (▲, Fig. 4.37), which can be deconvoluted into a fast initial increase with a rate constant 0.066 min\(^{-1}\) in the first 20 min and a slower rate with a rate constant the same as the decay of the nc form (0.0095 min\(^{-1}\)), the former is probably partially attributed from the cleavage of the sc form as seen during the initial stage of the reaction (lane 1). The initial concentration of the nc form is slightly greater than the final concentration of the ln form. This difference suggests that additional pathway(s) may be present for the disappearance of the ln form, such as further cleavage to yield small fragments that cannot be revealed on the gel.

Cu\textsuperscript{II} ions can cleave plasmids in the presence of an oxidation agent,\textsuperscript{105} thus serve as the reference herein (lanes 1’–6’). Cu\textsuperscript{II} cleaves the sc and nc forms into ln form less effectively than Cu\textsuperscript{II}-P1. The ln form is not apparent until about 20 minutes later (lane 2’), resulting in accumulation of the nc form. A simultaneous fitting of the time-dependent changes of both nc and ln forms to a consecutive kinetic pattern, i.e., $sc \rightarrow nc$
\[ \text{ln} \] (wherein the disappearance of the \textit{sc} form cannot be clearly traced within the time frame) reveals that the formation of \textit{nc} and \textit{ln} forms up to 60 min can be reasonably fitted (dotted trace, Fig. 1) without considering ds cleavage of the \textit{sc} form. A drastic change after 60 min (lanes 4′–6′) on both forms may reflect their quick degradation due to random multiple-site nicking to yield fragments as shown by the smeared gel pattern. The results reveal that Cu\textsuperscript{II}-\textbf{P1} shows apparent preference to nick and cleave \textit{sc} ds-DNA (to afford \textit{nc} and \textit{ln} forms, respectively) and to cleave \textit{nc} form into \textit{ln} form, rather than random cleavage toward all forms of plasmid as in the case of DNA cleavage by hydroxyl radicals which cannot be described by the kinetic patterns above. The cleavage pattern is reminiscent of Cu\textsuperscript{II}-bleomycin\textsuperscript{106} and some chemical model systems\textsuperscript{107} that have been demonstrated to exhibit ds-DNA cleavage. The apparent ds-DNA cleavage activity of Cu\textsuperscript{II}-\textbf{P1} may be attributed to the fact that this macromolecular complex does not quickly diffuse away from the reaction site after one DNA strand is cleaved. Thus, subsequent cleavage of the second strand can proceed effectively and becomes apparent in the early stage of the reaction (lanes 1 and 2). Moreover, the local [Cu\textsuperscript{II}] in Cu\textsuperscript{II}-\textbf{P1} is high which may result in localized cleavage. The copolymer itself and H\textsubscript{2}O\textsubscript{2} separately did not exhibit noticeable effect on the plasmid in two hours under the same conditions.

The oxidative DNA cleavage by this metallopolymer has also been determined to be effective compared to other systems.\textsuperscript{108-110} Linearization and further cleavage of plasmid DNA was observed in 30 min by a dinuclear Cu\textsuperscript{II} complex of 5 \textmu M in the presence of 3-mercapto-propionic acid, while only partial nicking of \textit{sc} plasmid was observed by a mononuclear Cu\textsuperscript{II} complex under the same conditions.\textsuperscript{108} Supercoiled plasmid was nicked by 20-\textmu g Cu\textsuperscript{II}-polymer complex in 10 min in the presence of monoperoxyphthalate.\textsuperscript{109}
Linearization of plasmid DNA by 10-µM Cu$^{II}$ in the presence of 25-µM 2-t-butyl(1,4)hydroquinone was reported to occur in 30 min.$^{110}$ The results show that Cu$^{II}$-P1 can serve as an efficient and selective “artificial ds-DNase” toward the cleavage of $sc$ and $nc$ ds-DNA. Since ds-DNA cleavage is a key to trigger cell apoptosis$^{53}$ the observation of the ds-DNase activity of Cu$^{II}$-P1 points a direction for rational design of DNA-targeting drugs for therapeutic purposes, e.g., clustered redox-metal centers with a high local metal concentration to afford a high ds-DNase activity.
Figure 4.38. Time course (10, 20, 40, 60, 90, 120 min, lanes 1-6 and 1’-6’, respectively) of plasmid cleavage by Cu$^{II}$-P1 in the presence of 1.5% H$_2$O$_2$ (lanes 1-6, 5.0 µM RU; ●, nc form; ▲, ln form; and theoretical fittings, solid and dashed traces) and by free Cu$^{II}$/H$_2$O$_2$ (50.0 µM, lanes 1’-6’; ○, nc form; △, ln form; and theoretical fitting, dotted traces) at pH 8.0 and room temperature, stained with ethidium bromide. The standard DNA ladder starts with 1 kbp from the bottom with 1 kbp increment upward. R is the plasmid DNA pQE30Xa of ~3.5 kbp without treatment. The data are fitted with the kinetic models discussed in the text.
7. Molecular Mechanics Calculations and Mechanism

The sequence and structure of a copolymer chain is random in nature and best described in terms of repeating units (RU). To gain some insight into the coordination sphere of CuII-P1 molecular mechanics calculations was used (MM3 force field,111 BioCACh 6.0 program, Fujitsu, Beaverton, OR), which reveal that the best coordination sphere is formed by pyridine ligands from different RUs. Metal binding to pyridines from the same RU causes significant distortion of metal-pyridine coordination, whereas binding to pyridines from different polymer chains afford a more preferred binding mode (~400 kcal/mol lower). The latter causes cross linkage of the polymer chains and results in coagulation of metallopolymer complexes at mM concentrations as we observed previously.112 The results discussed in the above sections suggest possible formation of a dinuclear active site in the oxidation catalysis.

The metal sites can be brought close to each other to form dinuclear sites analogous to that of hemocyanin with a pseudo-\(C_{2h}\) symmetry113 (Fig 4.38, top). This hemocyanin-like dinuclear site allows substrate binding to one of the metal ions from the top or bottom of the Cu-O\(_2\)-Cu plane without distorting the overall coordination sphere. A catechol substrate can be docked into the dinuclear site of peroxide-bound CuII-P1 without significant distortion (Fig. 4.38, middle), in which catechol is bound to one Cu center through a deprotonated hydroxyl while the other hydroxyl forms a H-bond with the bridging peroxide and a proximal amide group from the polymer. Upon deprotonation and binding of the second hydroxyl group to the other Cu center (Fig. 4.38, bottom), the energy is lowered relatively by ~240 kcal/mol. This binding mode has been suggested to be the case in the enzyme-substrate complex of catechol oxidase.113
However, the coordination is significantly distorted from the original, in which the Cu-O$_2$-Cu plane is puckered to the opposite direction.
Figure 4.39. (Top) Coordination structure of a putative dinuclear site in Cu$^{II}$-P1 calculated with the MM3 force field protocol. The di-copper site in hemocyanin (blue) is superimposed onto the structure to show their similarity. The Cu ions are shown in cyan and the bridging peroxo in red. (Middle) Docking of catechol (blue ball and stick) to the dinuclear site of Cu$^{II}$-P1 reveals a favourable binding configuration, in which catechol is bound to one Cu$^{II}$ through a deprotonated hydroxyl while the other hydroxyl is still protonated and H-bonding with the bridging peroxide and a proximal amide group from the polymer. (Bottom) Catechol is bound to the two metal ions upon deprotonation of the second hydroxyl group, which causes distortion of the di-Cu$^{II}$ center.
It has been shown that the metallopolymer Cu(II)-P1 can catalyze the oxidation of catechol type of substrates and neurotransmitters in the presence of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \) and the oxidative DNA cleavage in presence of \( \text{H}_2\text{O}_2 \) very efficiently. The mechanism proposed for the oxidation activity by Cu(II)-P1 is given in Figure 4.39 and it is analogous to what has been proposed for the oxidation of catechol type of substrates by catechol oxidase and tyrosinase as described in Fig. 4.3, section 4.1.

As shown in section 4.3, the oxidation can take place in presence of oxygen and in the presence of \( \text{H}_2\text{O}_2 \). According to the proposed reaction scheme (Fig. 4.39) a diphenol substrate can bind to the dinuclear center upon deprotonation (path A), supported by the saturation kinetics observed for all the examined catechol type of substrates, followed by oxidation to form the first quinone and the reduced state of the catalyst (path B) which can subsequently bind dioxygen and generate the oxy state (path C). A second diphenol molecule can bind to the \( \mu-\eta^2:\eta^2 \) \([\text{Cu(II)}_2\text{O}_2]^{2-}\) (path E) supported by molecular mechanics calculations (Fig. 4.38). Upon oxidation and formation of the second quinone molecule, the met state of the catalyst is generated and the catalytic cycle is complete. The reaction can also take place in \( \text{H}_2\text{O}_2 \) following the ‘shunt’ pathway (D and H) thus generating the same intermediates as in pathways C and F. The intrinsic \( (K_{\text{THB}} \text{ and } K_{\text{H2O2}}) \) and apparent dissociation constants \( (K_{\text{THB}} \text{ and } K_{\text{H2O2}}) \) are also shown in each pathway demonstrating that the oxidation substrates and \( \text{H}_2\text{O}_2 \) can bind independently and in the presence of each other.
Figure 4.40. Proposed random bi-substrate mechanism for the oxidation of THB by Cu$^{II}$-P1 in the presence (steps D–G or A,H,G) and absence (A–C and E–G) of H$_2$O$_2$. The production of H$_2$O$_2$ under reduction conditions (I) is also consistent with this mechanism. The substrate complexes at steps E and F are also shown in Fig. 4.38. The kinetic parameters can be obtained from the secondary plots of the Hanes equation.
8. Hydroxylation of phenolic substrates by Cu(II)-P1

The hydroxylation activity of Cu(II)-P1 complex was examined for the substrates phenol and tyramine in the absence and presence of H₂O₂. Furthermore, the initial rate of the hydroxylation of the chlorinated phenols 4-chlorophenol and 2,4,6-trichlorophenol was determined spectrophotometrically by examining the rate of o-quinone production and the amount of chloride ion released using gravimetric analysis.

Both phenol and tyramine exhibit an initial oxidation rate that is non-linear when plotted against increasing concentration of substrate, indicating that they follow pre-equilibrium kinetics as exhibited by the catechol containing substrates discussed in the previous section. The Michaelis-Menten kinetic parameters for the oxidation of phenol and tyramine were determined using 50 µM Cu(II)-P1 in 0.1 M HEPES pH 8.0 in 50% methanol in order to keep the catalyst soluble in the reaction medium and also enable the solubilization of the oxidation products, at 25 °C. The data are presented in Figures 4.40 through 4.43 and summarized in Table 4.9. The kinetic parameters for each substrate show that the hydroxylation rate by Cu(II)-P1 is dependent on the structure of the substrate used. The first and second order rate constants of phenol are \( k_{\text{cat}} = 0.0113 \text{ s}^{-1} \) and \( k_{\text{cat}}/K' = 6.5 \text{ M}^{-1}\text{s}^{-1} \) respectively under saturating amounts of H₂O₂ (20 mM). The rate accelerations of phenols oxidation in the absence and presence of 20µM Cu(II)–P1

Since both phenol and H₂O₂ are showing saturation they can both be considered substrates. The data was further analyzed with the Hanes plot (Eq. 4, section 4.2.1). The data in Figure 4.44 were fitted to a two-substrate random-binding mechanism according to Eq.6 (section 4.2.4), wherein the binding of phenol and H₂O₂ to the active center was assumed to be random and in rapid-equilibrium with a subsequent ordered product
release. Plotting of the slope and the y-intercept from in Hanes plots versus \(1/[\text{H}_2\text{O}_2]\) yield \(K_a\) (0.5 mM) and \(K_b\) (2.4 mM), the apparent values for the virtual dissociation of phenol and \(\text{H}_2\text{O}_2\), respectively in the presence of both substrates and the intrinsic dissociation constant for phenol is \(K_{\text{ia}}\) (0.97 mM). The \(K_{\text{app}}/K'\) ratio for phenol is 2.5 >1 which indicates a small effect on the dissociation of substrate in the presence of \(\text{H}_2\text{O}_2\).\(^{25}\)

The oxidation of a constant amount of phenol (10.0 mM) by 50 \(\mu\text{M}\) RU of Cu\(^{2+}\)-P1 as a function of \([\text{H}_2\text{O}_2]\) is also not linear (Fig. 4.43), which can be fitted to Eq.4 (section 4.2.3) to give \(k_{\text{cat}} = 0.0116\ \text{s}^{-1}\) and \(K'_{\text{H}_2\text{O}_2} = 3.64\ \text{mM}\). A noticeable oxidation of phenol by Cu\(^{II}\)-P1 was still observed at \([\text{H}_2\text{O}_2]\) = 0 mM, which was significantly higher than the auto-oxidation rate of phenol in the absence of Cu\(^{II}\)-P1 under the same conditions (\(k_o = 3.3 \times 10^{-10}\ \text{s}^{-1}\)). Thus, the oxidation of phenol by Cu\(^{II}\)-P1 was further investigated aerobically in the absence of \(\text{H}_2\text{O}_2\), wherein \(\text{O}_2\) serves as the oxidation agent in the reaction. Here, the rate of oxidation as a function of [phenol] is not linear (●, Fig. 4.40) which can be well fitted to pre-equilibrium kinetics (Eq. 4), exhibiting rate constants \(k_{\text{cat}} = 1.55 \times 10^{-3}\ \text{s}^{-1}\) and \(K' = 1.25\ \text{mM}\), and an apparent second-order rate constant \(k_{\text{cat}}/K' = 1.24\ \text{M}^{-1}\ \text{s}^{-1}\). The first-order rate accelerations of phenol oxidation in the absence and presence of 20.0 mM \(\text{H}_2\text{O}_2\) exhibit significant magnitudes of \(6.0 \times 10^5\) and \(7.8 \times 10^4\)-folds, respectively, with respect to the auto-oxidation of phenol under the same conditions without the catalyst.
Figure 4.41. Saturation profiles of phenol hydroxylation in various $[\text{H}_2\text{O}_2]$ by 50 µM Cu(II)--P1 in methanol–buffer pH 8.0 HEPES 0.1M in 50% methanol (top) and Hanes analysis plot (bottom).
**Figure 4.42.** Hydroxylation of phenol by 50 µM Cu(II)-P1 in 50% methanol at pH 8.0 as a function of [H$_2$O$_2$]
The hydroxylation activity of Cu(II)-P1 was also checked towards tyrosine but the rate was not easily observable and this slow reactivity was attributed to the structure of tyrosine bearing an electron withdrawing carboxylate on the aliphatic chain of the molecule. To confirm this hypothesis, tyramine was also used as a substrate for comparison since in vivo, removal of the carboxylate group of tyrosine leads to the formation of tyramine (Fig. 4.42). The hydroxylation of tyramine proceeded with observable rates and activities comparable to the rates of phenol hydroxylation under similar conditions (Figure 4.43). The first and second order rate constants of tyramine are $k_{\text{cat}} = 9.0 \times 10^{-5}$ s$^{-1}$ and $k_{\text{cat}}/K' = 0.012$ M$^{-1}$ s$^{-1}$ respectively, in air and $k_{\text{cat}} = 7.2 \times 10^{-2}$ s$^{-1}$ and $k_{\text{cat}}/K' = 9.0$ M$^{-1}$ s$^{-1}$ in presence of saturating amounts of H$_2$O$_2$ (100 mM). The turnover numbers for tyramine in comparison to phenol in air and in H$_2$O$_2$ is ten time faster (Table 4.9) supporting a selectivity in substrate binding for hydroxylations reactions catalyzed by Cu(II)–P1.
Figure 4.43. Comparison of the hydroxylation of tyramine (●) and tyrosine (○) by 50 μM Cu(II)-P1 in 100 mM H₂O₂ at pH 8.0 in 50% methanol.
Figure 4.44. Hydroxylation of tyramine by 50µM RU of Cu$^{2+}$-P1 in presence of 0mM($\bullet$), 5mM($\circ$), 12mM ($\blacksquare$), 50 mM ($\bullet$) and 100 mM ($\blacktriangledown$) H$_2$O$_2$ at pH 8.0 (top) and Hanes analysis (bottom).
Figure 4.45. Hydroxylation of Serotonin by 25mM Cu(II)-P1 at pH 8.0 in presence (○) and absence (●) of H₂O₂.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$M$^{-1}$)</th>
<th>$k_o$ (s$^{-1}$)</th>
<th>Krel</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.26</td>
<td>$1.55 \times 10^{-3}$</td>
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<td>$3.3 \times 10^{-9}$</td>
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<td>tyramine</td>
<td>7.4</td>
<td>$9.0 \times 10^{-5}$</td>
<td>0.012</td>
<td>$2.1 \times 10^{-8}$</td>
<td>$4.3 \times 10^3$</td>
</tr>
<tr>
<td>serotonin</td>
<td>0.45</td>
<td>$2.5 \times 10^{-4}$</td>
<td>0.5</td>
<td>$1.2 \times 10^{-7}$</td>
<td>$2.1 \times 10^3$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$M$^{-1}$)</th>
<th>$k_o$ (s$^{-1}$)</th>
<th>Krel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>1.74±0.29</td>
<td>$0.012\pm5.1 \times 10^{-4}$</td>
<td>6.5</td>
<td>$3.3 \times 10^{-8}$</td>
<td>$3.4 \times 10^5$</td>
</tr>
<tr>
<td>tyramine</td>
<td>8.0±0.93</td>
<td>(7.2±0.88) $\times 10^{-2}$</td>
<td>9.0</td>
<td>$2.2 \times 10^{-7}$</td>
<td>$3.2 \times 10^5$</td>
</tr>
<tr>
<td>serotonin</td>
<td>1.2±0.22</td>
<td>(1.4±0.23) $\times 10^{-3}$</td>
<td>1.1</td>
<td>$1.2 \times 10^{-6}$</td>
<td>$1.2 \times 10^3$</td>
</tr>
</tbody>
</table>

**Table 4.10.** Kinetic parameters for the hydroxylation of several substrates in air (top) and in presence of saturating $H_2O_2$
Gradual replacement of Cu$^{2+}$ in Cu-P1 with redox-inactive Zn$^{2+}$ is expected to serve as a practical method for addressing the nature of the active center, such as cooperativity, by steady inactivation of the catalyst through the dilution with Zn$^{2+}$. A linear correlation between the activity and the extent of Zn$^{2+}$ dilution should be observed for metal binding to RU with a 1:1 stoichiometry if there is no cooperativity and/or interactions between/among different Cu$^{2+}$ active sites. Quite the opposite, a sigmoidal activity profile was observed as a function of the mole fraction of Cu$^{2+}$ in P1 toward phenol oxidation in the presence of 10 mM H$_2$O$_2$, which can be fitted to the Hill equation (Eq. 4) with a significant Hill coefficient $\theta = 4.34$ and $r^2 = 0.99$ (Fig. 4.48). The activity profile for phenol oxidation in this experiment without H$_2$O$_2$ also shows a sigmoidal trace, however, with a smaller $\theta$ value of 1.09 and $r^2 = 0.99$. This observation suggests that a cooperative di-Cu$^{2+}$ active center maybe present in the oxidation of phenol by Cu$^{2+}$-P1 in the presence of H$_2$O$_2$, which is consistent with the catalytic cycle of tyrosinase.$^{114}$ Such cooperative dinuclear center is not strongly implied in the aerobic oxidation of phenol by Cu$^{2+}$-P1 in the absence of H$_2$O$_2$, which must have a different reaction pathway. The mechanisms of the hydroxylation of phenol in the presence and absence of H$_2$O$_2$ are further addressed.
Figure 4.46. Hydroxylation of 8.0 mM phenol catalyzed by 60 µM RU of Cu-P1 as a function of Cu$^{2+}$ mole fraction at a constant [Cu$^{2+}$] + [Zn$^{2+}$] of 60 µM in the absence (●) and presence (○) of 10.0 mM H$_2$O$_2$. 
10. Kinetic Isotope Effect

The rate of hydroxylation of phenol can potentially be affected by the presence of a C–D bond in place of a C–H bond, if the C–H bond cleavage is the rate determining step. The kinetic isotope effect (KIE) for the hydroxylation of phenol was probed using deuterated phenol in the absence of H$_2$O$_2$ and in presence of saturating amount of peroxide (20 mM) yielding KIE values of 1.19 and 2.80 respectively (Fig. 4.46). The small KIE value in the absence of H$_2$O$_2$ suggests that the cleavage of the C–H bond is not the rate determining step for this pathway. The differences observed in the KIE for the hydroxylation in the presence and absence of H$_2$O$_2$ suggest that the rate determining step under these two different conditions must also be different, which is consistent with the results from the “Zn$^{II}$” dilution shown in section 4.6.1.

11. Mechanistic Job Plot

To further support the existence of a dinuclear center responsible for the observed activity a “mechanistic Job plot” was conducted. The classic optical-based Job plot (absorption vs. mole fraction)$^{115}$ determines the stoichiometry of a metal complex that is formed in one equilibrium $\text{M + nL} \leftrightarrow \text{ML}_n$, wherein the maximum absorption affords the stoichiometry in terms of mole fraction $X$. Thus, a maximum absorption at $X_M = 0.5$ reflects the formation of a M:L = $X_M:X_L = 1:1$ complex, whereas a maximum absorption at $X_M = 0.67$, a M:L = $X_M:X_L = 2:1$ complex. In the case of pre-equilibrium kinetics shown in Eq. 1 (section 4.2.3), the formation of the intermediate $\text{P1-Cu}^{II}$-S is the determinant for the activity and is under equilibrium with the reactant analogous to the equilibrium for the formation of metal complexes.
Figure 4.47. Kinetic isotope effect in phenol hydroxylation by 50 µM Cu-P1 in air (top) and in 50 mM H₂O₂ (bottom) at pH 8.0 with 0.1 M HEPES in 50% methanol. Deuterated phenol (●) and regular phenol (○)
Thus, the maximum activity in the “mechanistic Job plot” reflect the stoichiometry of the ratio S:Cu\textsuperscript{II}-P\textsubscript{1}. Thus, a maximum activity at $X_{\text{Cu-P1}} = 0.5$ reflects the formation of 1:1 S:Cu\textsuperscript{II}-P\textsubscript{1} intermediate whereas a maximum activity at $X_{\text{Cu-P1}} = 0.67$ indicates the formation of a dinuclear active center in the intermediate as S–(Cu\textsuperscript{II}-P\textsubscript{1})\textsubscript{2}. The difference in stoichiometry of the intermediate is clearly revealed in the presence and absence of H\textsubscript{2}O\textsubscript{2} with the maximum at $X_{\text{Cu-P1}} = 0.67$ and 0.5, respectively (Fig. 4.47). A cooperative pattern seems to be also present (dotted trace). These results reflect that a dinuclear-Cu\textsuperscript{II} intermediate may be present at the rate-determining step in the former case while it is more likely a mononuclear-Cu\textsuperscript{II} intermediate at the rate-determining step without H\textsubscript{2}O\textsubscript{2}. The results are consistent with the “Zn\textsuperscript{II} dilution” results (Fig. 4.45) wherein the apparent cooperativity hints at a possible di-metal active center, and the KIE (Fig.4.46)
Figure 4.48. Mechanistic Job Plot for phenol hydroxylation by Cu$^{II}$-P1 in the presence (●) and absence (○) of 80 mM H$_2$O$_2$ at pH 8.0, wherein the solid traces are the best fit to metal-to-substrate ratio ([ML]:[S]) of 2:1 and 1:1, respectively, while the dashed trace represents [ML]:[S] ratio of 1:1 which does not fit the data.
12. Effect of Sodium Borate

To show that the observed activity is only dependent on the hydroxylation of the phenol ring and that there is no release and accumulation of intermediate catechol during the reaction, the activity was checked in the presence of various amounts of sodium borate that is known to interact with syn-diol-containing molecules such as catechol. A decrease in activity would be observed if catechol is released during the reaction (which would decrease the amount of $o$-quinone). A decrease in activity would be observed if catechol was released during the reaction. The result confirms that the hydroxylation rate remains virtually unchanged indicating that orthoquinone is the only product released in the reaction. To further support this claim, the oxidation of catechol by Cu$^{II}$-P1 was measured under the same conditions and in the presence of the same amounts of borate. The inhibition of the catechol oxidation by borate indicates that if catechol was released during the reaction the presence of borate would be inhibitory. Results are shown in Fig. 4.48.
Figure 4.49. Effect of sodium borate on the hydroxylation of 5 mM catechol or phenol (inset) at pH 9.0 by 10 µM Cu(II)–P1
13. Effect of pH on hydroxylation reaction

In order to understand how the acidity can affect the rate of the hydroxylation and to determine the number of ionizable groups important in the observed catalysist that are present in the reaction, the oxidation rate of phenol hydroxylation was determined under different pH. The hydroxylation of 8.0 mM phenol in air and in the presence of 10 mM H$_2$O$_2$ by 50 µM Cu(II)-P1 at pH 8.0 in 50% methanol was determined in the pH range of 5.5-11.0. Both plots are sigmoidal with pK$_a$ values of 8.3 and 8.5 respectively. The similarity of the pKa values suggests that they could correspond to the same ionizable group.

![Effect of pH on the hydroxylation of 8 mM phenol by 50 µM Cu(II)–P1 methanol–buffer pH 8.0 HEPES 0.1M IN 50% methanol in air (●) and in 10 mM H$_2$O$_2$ (○).](image)

**Figure 4.50.** Effect of pH on the hydroxylation of 8 mM phenol by 50 µM Cu(II)–P1 methanol–buffer pH 8.0 HEPES 0.1M IN 50% methanol in air (●) and in 10 mM H$_2$O$_2$ (○).
14. Molecular Mechanics Calculations for phenol binding to Cu(II)–P1

Molecular mechanics calculations with the MM3 force field was used as discussed in section 4.5 to probe the coordination environment of the metal ions in the presence of P1. Despite geometric constraint, the metal coordination can be brought close to each other to form a dinuclear site upon peroxo binding (Fig. 4.51, top) to exhibit a coordination sphere analogous to the pseudo-$C_{2v}$ symmetry of the oxygen-transport protein hemocyanin with a $\eta^2,\eta^2-\mu$-peroxo-di-Cu$^{II}$ center (blue ball-and-stick structure). This hemocyanin-like dinuclear site allows substrate binding to one of the metal ions from the top or bottom of the Cu-O$_2$-Cu plane without distorting the overall coordination sphere. A puckered di-Cu$^{II}$-peroxo geometry is slightly more stable than a planar geometry by 23 kcal/mol. A phenol molecule can be brought to the dinuclear site of Cu$^{II}$-P1 without significant disturbance of the dinuclear site (Fig. 4.51, bottom) which further lowers the energy by 170 kcal/mol, wherein phenol is bound to one Cu center in the form of phenolate which in turn is H-bonded with a proximal amide group from the polymer. Such a configuration brings the ortho carbon of the bound phenol within 3.8 Å from one of the oxygen atoms of the bridging peroxide.
**Figure 4.51.** (Top) coordination structure of dinuclear site in Cu(II)−P1 calculated on the basis of MM3 force field protocol. The di−Cu site in hemocyanin (blue) is superimposed onto the structure to reveal their similarity. The cu ions are shown in cyan and the bridging peroxo in red (bottom) Phenol (pink) is bound to one Cu center as phenolate and forms an H−bond with a proximal amide group from the polymer.
15. Catalytic mechanism for hydroxylation of phenolic substrates

The kinetic results described herein strongly suggest that the oxidation of phenol by Cu$^{2+}$-P1 in the presence of H$_2$O$_2$ follows the reaction mechanism of the di-Cu Type-3 enzyme tyrosinase$^{4,114}$ wherein an active $\mu$-$\eta^2,\eta^2$-peroxo-di-Cu$^{2+}$ center is formed (Fig. 4.52, B) which can perform hydroxylation of phenol (B–D). Here, the binding of phenol to the active center should be a fast step, followed by slow peroxo attack and C–H bond breakage to exhibit deuterium KIE. Conversely, the binding of phenol to a mononuclear Cu$^{II}$ center is expected to occur in the absence of H$_2$O$_2$ (E), which is followed by an internal electron transfer to form a phenol radical (F). This should be the slowest step under equilibrium to exhibit a mononuclear character (Fig. 4.47) without showing a significant KIE (Fig. 4.46), which is followed by dioxygen binding and electron transfer to form a Cu$^{II}$–O$_2^-$ center (G). Once it is formed, it should be followed by a fast coupling of the phenol and superoxide radicals, then C–H bond breakage to form the product (D). The latter steps may be assisted by a dinuclear center.

The activity of the metallopolymer Cu$^{II}$-P1 in the presence of the green oxidation agents H$_2$O$_2$ and O$_2$ toward the hydroxylation and oxidation of phenol is quite proficient. Moreover, the easy synthesis of P1 and analogous copolymers and their metal complexes suggests that metallopolymer systems can serve as an excellent template for further design of metallopolymers for chemical, biological, and environmental applications.
Figure 4.52. Proposed mechanism for the oxidation of phenol by Cu\textsuperscript{II}-P1 in the presence (A–D) and absence (E–G and C–D) of H\textsubscript{2}O\textsubscript{2}.
16. EPR characterization of the dinuclear center

Electron paramagnetic resonance spectroscopy (EPR) is a technique that can help elucidate certain properties and the environment of a paramagnetic center.\(^{117}\) This is achieved by characterization of the interaction of the paramagnetic center with an applied magnetic field. The EPR spectra of Cu(II)--P1 complexes in the presence of Zn(II) was obtained at different equivalents of Cu(II): Zn(II). The rationale of this experiment is that magnetic dilution of Zn(II) can lead to the disappearance of any hyperfine features in the spectra of the Cu(II)--P1 due to the ligand environment, since if the metal center is dinuclear, coupling of the dicopper center would lead to a diamagnetic complex. The disappearance of the hyperfine coupling at \(g//\) is indicative of magnetic coupling of Cu(II) ions which may be due to the proximity of Cu(II) ions bound to the polymer structure. Nevertheless, the coupling between the two Cu(II) centers is visible even at 0.5 Cu(II):0.5 Zn(II) (Fig. 4.52).

The half field signal (Fig. 4.53) is also indicative of the presence of magnetic coupling, suggesting possible dinuclear center. These findings provide physical evidence to further support the proposed mechanism for the observed catalysis by the Cu(II)--P1 complex.
Figure 4.53. EPR spectra of Cu(II)-P1 in the presence of different molar ratios of Zn(II) obtained at 6 K on a Bruker ELEXSYS E580 FT/CW EPR spectrometer equipped with an Oxford CF-935 cryostat (at the University of Florida).
Figure 4.54. Comparison of EPR spectra in lower field region for Cu(II):Zn(II) 1:1 ratio and in Cu(II) in presence of one equivalent of P1 in 50% MeOH
17. Natural antioxidants and inhibition studies

Flavonoids are naturally occurring phenolic compounds that have been extensively studied for their antioxidant properties. Their structure–antioxidant activity relationships have been studied but what is lacking in the field is a thorough understanding of the similarities and the differences in the mechanism of action among the different classes of natural antioxidants. Inhibition of an efficient oxidation agent such as Cu(II)–P1 using natural antioxidants can provide an alternative model to study the antioxidant activities with possible implications in oxidative stress in biological systems. It was shown (section 4.3.4) that Cu(II)–P1 catalyzes the efficient oxidation of dopamine, so in this study we use this as substrate in presence of a natural antioxidant. Quercetin shows high inhibition that is consistent with literature references on this compound. Other potential inhibitors of the observed oxidation includes Catechin and ellagic acid. From the plot of the data (Fig. 4.51) it is concluded that all these compounds are potential inhibitors for dopamine or other neurotransmitters. The detailed mechanism of their activity and the extent of substrate protection caused by natural antioxidants must be further determined.
Figure 4.55. Inhibition of oxidation of 5 mM dopamine by natural antioxidants (+)-catechin, quercetin and ellagic acid. The amount of Cu(II)-p1 used is 10 µM and is performed in 0.1 M buffer MES Ph 6.0 in 50% methanol
18. Oxidative dechlorination of polychlorinated aromatic compounds

The oxidation of chlorinated phenols (Fig. 4.8–4.9 for structures) yielded more than one oxidation products and as it can be seen in Fig. 4.5, the distribution of the products depends on the amount of chlorine substitutions on the phenolic ring. These findings suggest that there is a possibility of a multistep mechanism leading to more than one distinct products. One of the observed peaks can be attributed to an ortho-quinone product since the reactions were performed in the presence of MBTH which forms a colored adduct at 500 nm (Fig. 4.55).

The results indicate that the catalyst was able to significantly enhance the decomposition of these chlorophenols (Fig. 4.56–4.58). An initial estimation of the rates from the Absorption / time plots indicates that the rates of oxidation products formation is ~20 times faster. The amount of chloride present in the reaction after 10 min reaction was determined for all the above samples. It was found that the samples that lacked the catalyst didn’t produce any Cl- in solution (determined by silver titration according to Volhard’s method). One important consideration for this application to be further explored is the determination of the identity of the oxidation products in order for the
(a) Product formation profile during oxidation of 5 mM 2,4,6 TCP by 50 µM Cu(II)-P1 and 10 mM H2O2 at pH 8.0

(b) Product formation profile during oxidation of 5 mM 2,4 Dichlorophenol by 50 µM Cu(II)-P1 and 10 mM H2O2 at pH 8.0.
(c) Product formation profile during oxidation of 5 mM 4-Chlorophenol by 50 µM Cu(II)-P1 and 10 mM H2O2 at pH 8.0.

(d) Just 4-Chlorophenol oxidation at pH 8.0 (same conditions as the Cu(II)-P1 samples).
(e) Just 2,4-Dichlorophenol oxidation at pH 8.0 (same conditions as the Cu(II)-P1 samples).

(f) Just 2,4,6-Trichlorophenol oxidation at pH 8.0 (same conditions as the Cu(II)-P1 samples).

**Figure 4.56.** Oxidation of 4-chlorophenol, 2,4- dichlorophenol and 2,4,6 trichlorophenol in the presence ((a)-(c)) and in the absence of the Cu(II)-P1 catalyst.(d)-(f)
Figure 4.57. Time trace of oxidation products in 5 mM 4-chlorophenol oxidation without catalyst or in the presence of 50 mM Cu(II)–P1 at pH 8.0 in 50% methanol
Figure 4.58. Time trace of oxidation products in 2, 4−dichlorophenol oxidation without catalyst or in the presence of 50 μM Cu(II)−P1 at pH 8.0 in 50% methanol
Figure 4.59. Time trace of oxidation products in 2, 4, 6–trichlorophenol oxidation without catalyst or in the presence of 50 µM Cu(II)–P1 at pH 8.0 in 50% methanol.
IV. Concluding Remarks and Future Perspectives

One of the most significant intermediates in oxidations and hydroxylation reactions in the biological systems is the $\mu-\eta^2-\eta^2$-peroxo-dicopper intermediate which has been implicated in the catalytic cycle of tyrosinase and catechol oxidase. This intermediate has been studied extensively by the use of simple metal complexes of Cu(I) and Cu(II) of a variety of ligand systems (section 4.1.1). However, polymer-based systems capable of forming active dicopper intermediates are very scarce (only two examples in the literature out of which one is polymer based and the second is silica based) and the kinetic characterization of the observed activity is not extensive.

Most of the literature in the area of copper oxygen chemistry has been focusing on the spectroscopic characterization of the active copper-oxygen intermediates under extreme conditions (very low temperatures and typically in organic solvents rather than aqueous environment). Furthermore, the extent of kinetic characterization of most of the catalytic reactions is limited. In this dissertation, the catalytic efficiencies of Cu(II)$-P1$ were determined for a series of catechol type of substrates in the presence of molecular oxygen with efficiencies reaching 10,000-fold higher than the reactions without the catalyst, and reaching 40,000-fold in $H_2O_2$ under very mild conditions (pH 6.0 in 50% methanol aqueous solution at room temperature). The Cu(II)$-P1$ polymer complex is also capable of hydroxylating phenolic substrates with efficiencies reaching $5.0 \times 10^5$-fold higher than the reactions without the catalyst both in the presence of molecular oxygen and in $H_2O_2$ which is among the highest compared to other scarcely reported model systems. Moreover, Cu(II)$-P1$ is unique in its ability to hydroxylate phenol in the presence of oxygen in the form of its Cu(II) oxidation state. In the case of tyrosinase, the
enzyme is inactive in its met-form (i.e., both copper centers are in the Cu(II) oxidation state), wherein phenol is considered inhibitory. Hydroxylation of phenols by met-tyrosinase does not occur unless a reducing agent is added in the presence of oxygen or H₂O₂ is added to produce the active dicopper-peroxo intermediate. This aspect of phenol oxidation is primarily responsible for the “lag period” that is characteristic of in vitro tyrosinase oxidations. This behavior is not observed in the case of Cu(II)–P₁ which suggests a distinct catalytic pathway in the presence of oxygen when compared to the catalytic cycle observed for H₂O₂ (sections 4.6.1–4.6.3) and also shows the versatility of the metallopolymers system undergoing effective hydroxylation catalysis under oxidative conditions that is not observed in tyrosinase catalysis.

The high oxidative activity of Cu(II)–P₁ in the presence of oxygen or hydrogen peroxide suggests that it can be used in catalytic oxidations of phenolic substrates containing various substituents. Chlorinated phenols are more difficult to oxidize due to the electron withdrawing character of the chlorine substituents on the aromatic ring. However, I described in this dissertation that Cu(II)–P₁ is capable of oxidizing a series of chlorinated phenols. According to the preliminary results, there is a distribution of products as shown by the UV–vis spectra of the reaction mixture over time (section 4.9). Identification of the structure of these products could aid in determination of the reaction mechanism for the observed oxidations. Nevertheless, the formation of free Cl⁻ ions in the reaction medium suggests that the oxidation of the chlorinated substrates proceeds with the release of the chlorine substituents, leading to products that are less environmentally toxic. The detailed catalytic mechanism and identifying the most suitable Cu(II)–polymer complexes for oxidation of chlorinated substrates must be
further explored in the future which may have broad applications in environmental remediation.
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