ALKALINE PHOSPHATASE SENSORS BASED ON AMPLIFIED QUENCHING OF CONJUGATED POLYELECTROLYTES

By

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To my parents
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We investigated chemiluminescent and fluorescent sensors for alkaline phosphatase (ALP) based on the amplified quenching of functionalized poly(para-phenylene ethynylene)s, including poly(phenylene ethynylene) carboxylate and a cationic poly(phenylene ethynlenes) which has dendritic charged amino groups. Although they relied on two different emission methods, these two sensors shared the same quenching-unquenching mechanisms and both featured high sensitivity and good selectivity.

First, a chemiluminescent sensor was developed to detect ALP based on the amplified quenching of PPE-CO$_2^-$ by Cu$^{2+}$ and the interaction between Cu$^{2+}$ and pyrophosphate, a substrate of ALP. A peroxyoxalate chemiluminescence (POCL) system which consists of bis(2,4,6-trichlorophenyl) oxalate (TCPO) as aryl oxalate, imidazole as catalyst, H$_2$O$_2$ as oxidant, and PPE-CO$_2^-$ as fluorophore has been developed and optimized. The quenching of chemiluminescence of PPE-CO$_2^-$ by Cu$^{2+}$ was more sensitive than that of fluorescence under the same conditions. This chemiluminescent sensor has been successfully applied to qualitatively and quantitatively detect ALP activity.

Second, a convenient fluorescent assay was developed to detect the pyrophosphatase activity of ALP based on the direct interaction between PPE-$^d$NH$_3$Cl and pyrophosphate. The
fluorescence of cationic dendritic PPE-\textsuperscript{d}NH\textsubscript{3}Cl was sensitive to anions with more negative charges including pyrophosphate, ATP and ADP, while not affected by anions with less negative charges including phosphate and AMP. The PPE-\textsuperscript{d}NH\textsubscript{3}Cl/PPi system has been employed to detect the enzymatic activity of ALP by monitoring the hydrolysis of pyrophosphate which induces rapid and sensitive fluorescence responses. The calibration plot was directly derived from the linear range of Stern-Volmer plot, which easily converted real-time fluorescence plots into enzymatic reaction processing curves. This ALP turn-on assay allowed the derivation of kinetic parameters and inhibition constant of phosphate for the ALP activities.
CHAPTER 1
INTRODUCTION

Conjugated Polyelectrolytes

Conjugated polymers (CPs) have been developed and widely used since Shirakawa et al.\textsuperscript{1} discovered in 1977 that polyacetylenes obtained unusual conductivity, as high as 10 million times, upon halogen doping. The stable charge-transfer $\pi$ complexes were believed to be formed during the halogen doping to achieve the systematically controllable electrical properties. As the first step of making plastics electrically conductive, this discovery led to the 2000 Nobel Prize in Chemistry, which was awarded to Hideki Shirakwa of the University of Tsukuba in Japan, Alan MacDiarmid of the University of Pennsylvania at Philadelphia and Alan Heeger of the University of California at Santa Barbara.\textsuperscript{2,3} The past decades witnessed revolutionary applications of conducting polymers, such as flat panel displays using OLEDs,\textsuperscript{4} light-emitting electrochemical cells (LECs),\textsuperscript{5} polymer solar cells,\textsuperscript{6} field-effect transistors (FETs),\textsuperscript{7} plastic lasers,\textsuperscript{8} and chemical and bio-sensors.\textsuperscript{9,10}

Conjugated polymers (CPs) are chain-like compounds with alternating double and single bonds as their backbones. CPs feature the fantastic optoelectronic/redox properties because whenever excess charges are on the polymer chains, the charges can hop along the conjugated backbones easily. Neutral CPs are normally wide band gap organic semiconductors that exhibit efficient absorption or emission at the band edge.\textsuperscript{1} The strong luminescence is related to the delocalization and polarization of the electronic structure. Due to the extraordinary photophysical and electrochemical properties induced by the chemical doping of CPs, many CPs (Figure 1-1) have been synthesized and investigated: polyacetylene,\textsuperscript{3} poly(para-phenylene) (PPP),\textsuperscript{11} poly(para-phenylene vinylene) (PPV),\textsuperscript{4,12} poly(para-phenylene ethynylene) (PPE),\textsuperscript{12} polythiophene (PT),\textsuperscript{7} and polypyrrole (PPy).\textsuperscript{13}
Conjugated polyelectrolytes (CPEs) retain the high absorption coefficient (excellent “light harvesting” properties) and high fluorescent quantum yields, which originate from their \( \pi \)-conjugated backbones.\(^{14}\) However, with attached ionic solubilizing side-chains, such as sulfonate (-SO\(_3^–\)), carboxylate (CO\(_2^-\)), phosphate (PO\(_3^{2-}\)) and alkyl ammonium (NR\(_3^+\)), CPEs are soluble in water and other polar solvents. CPEs feature amplified quenching effect credited both to strong association to small molecular quencher with opposite charges and to efficient exciton transport to quencher sites.\(^9\) These unique properties make CPEs attractive materials for highly sensitive fluorescence-based sensors for biological and chemical targets.\(^{15-17}\)

Poly(arylene ethynylene)s (PAEs) comprise an important segmental CPEs family. PAEs share the same backbone of conjugated ethynyl linked aromatic or heteroaromatic rings. Typically PAEs are insulators in the neutral state but became conductive by either oxidization or reduction of the polymer’s \( \pi \)-electron system. Their semiconducting properties have generated some interest in device applications of electroluminescent polymers. However, PAEs’ photophysical properties and corresponding applications in TNT detection\(^{18,19}\) and biological sensors\(^{20,21}\) make them one of the most important classes of conjugated polymers. Considered as
a molecular wire for exciton transport, PAEs are sensitive to small perturbations in their band structure and act as antennae for harvesting optical energy.²²

As a representative class of PAEs, PPEs have been well studied and applied to many sensory systems, including methyl viologen salt sensors,²¹,²³ TNT sensors,¹⁸,¹⁹ metal ion sensors,²⁴-²⁶ and PPI sensors.²⁷ The PPEs not only feature superior photostability compared to other CPEs, such as PPVs, but they also demonstrate different electronic and optical properties from parent molecules upon structural modifications. The main chains of PPEs have three isomers: ortho-, meta- and para-, defined by their different connectivity via the alkyne groups. Different aromatic building blocks can also be introduced into the conjugated backbone to engineer the electronic properties (Figure 1-2).²⁸ Also, variable side chains can be introduced to modify the polymer structures. All these strategies are meant to make the PPEs amphiphilic, water-soluble, self-assembling, and able to form helical structures or attached with receptors for targets.

Figure 1-2. Examples of PPEs with different structural modifications
Fluorescence Quenching

Fluorescence is widely used in chemical sensing not only because of its sensitivity but also because of the availability of the diverse transduction schemes, which are based on changes in fluorescence intensity, fluorescence lifetime, and excitation or emission wavelength. The main reason for employing CPEs in the chemical sensory scheme is their amplified fluorescence quenching response to small perturbations. There are usually two fluorescence quenching mechanisms: static quenching and dynamic quenching. Static and dynamic quenching both require molecular contact between the fluorophore and quencher. However, static quenching is due to the formation of a non-fluorescent complex between the fluorophore and the quencher. On the other hand, dynamic quenching, which is also called collisional quenching, is due to random collisions between fluorophore and quencher molecules. So for static quenching the quencher must diffuse to the fluorophore within the lifetime of the excited states.

Figure 1-3. Mechanism of fluorescence quenching. A) Dynamic quenching. B) Static quenching. C) Combination of dynamic and static quenching.
Figures 1.3a and 1.3b describe dynamic quenching and static quenching, respectively. In these figures, $F^*$ represents the excited fluorophore, $Q$ represents quencher, and $k_q$ is the bimolecular quenching rate constant.

There are several ways to distinguish these two mechanisms. First, the lifetime in static quenching does not change, because the fluorescence occurs from the uncomplexed fluorophore, which remains the same during the quenching process. However, the lifetime in dynamic quenching decreases in proportion to the intensity. Second, static quenching is decreased at higher temperature due to dissociation of weakly bound complexes formed in the quenching process, while dynamic quenching is increased at higher temperature due to faster diffusion and collision.

The Stern-Volmer (SV) equation is used to describe the emission intensity quenching:

$$\frac{I_0}{I} = 1 + K_{sv}[Q]$$  \hspace{1cm} (1-1)

where $I_0$ and $I$ are fluorescence intensities in the absence and presence of quencher, respectively; $[Q]$ is the quencher concentration; and $K_{sv}$ is the Stern-Volmer quenching constant. Both static and dynamic quenching can be expressed in terms of the SV equation. In dynamic quenching, the SV equation can be also written as:

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q]$$  \hspace{1cm} (1-2)

where $\tau_0$ and $\tau$ are the fluorescence lifetimes in the absence and presence of the quencher respectively. Here, $K_{sv}$ is replaced by $K_D$, which is equal to $k_q \tau_0$. For diffusion-controlled quenching, the bimolecular quenching constant $k_q$ cannot exceed the diffusion rate constant (ca. $10^{10}$ M$^{-1}$ s$^{-1}$). If $k_q$ is greater than $10^{10}$ M$^{-1}$ s$^{-1}$, usually static quenching is occurring. In static
quenching, $K_{sv}$ is represented by $K_a$ as shown in the following equation, which is the association constant for formation of the ground state complex $[F\cdot Q]$.

$$\frac{I_0}{I} = 1 + K_a [Q]$$  \hspace{1cm} (1-3)

where $K_a$ is the binding constant of the ground state association between the fluorophore and the quencher.

Figure 1-4. The S-V plot of the combined dynamic and static quenching.

When either static quenching or dynamic quenching dominates the quenching process, the SV plot of $I_0/I$ vs. $[Q]$ is linear according to equations 1-1 and 1-2. When static quenching dominates, the slope of the plot equals $K_a$; while when dynamic quenching dominates, the slope gives $K_D$. But in many cases fluorescence quenching involves both static and dynamic quenching mechanisms, as shown in Figure 1-3 the SV plots are nonlinear, upward-curved(Figure 1-4). A modified SV equation has been formulated to fit the quenching data in the upward-curving SV plots. It shows the combination of static and dynamic quenching effect on the relationship of $I_0/I$ and quencher concentration $[Q]$:

$$\frac{I_0}{I} = (1 + K_D [Q]) (1 + K_S [Q])$$
The upward-curving quenching is sometimes explained by “sphere of action”. This means that the fluorophore and quencher do not actually form a ground-state complex. Instead, when the quencher is adjacent to the fluorophore at the moment of excitation, these closely spaced fluorophore-quencher pairs are immediately quenched. So within the sphere of action, the probability of quenching is unity. In the fluorescence quenching of conjugated polymers, this effect is called “superlinear quenching,” which can arise from a variety of processes, including mixed static and dynamic quenching, variation in the association constant with quencher concentration, and chromophore (or polymer) aggregation.

**Amplified Quenching of Conjugated Polyelectrolytes**

The dominant attribute that has driven interest in CPE-based sensors is their ability to produce superior amplified signal gains in response to minor perturbations, compared to small molecule indicators. The amplified fluorescence quenching of CPs has been called the “molecular wire effect”, which was first described by Swager’s group in 1995. The authors believed that the amplification is a result of the ability of the CP’s delocalized electronic structure (i.e., energy bands) to facilitate efficient energy migration over large distances. To prove this proposal, the authors conducted parallel studies on neutral PPEs (2), featuring the bis(p-phenylene)-34-crown-10 (BPP) group on each repeating unit, as well as the corresponding monomer (1). Since BPP is an exceptional receptor of paraquat, a well-known electron transfer quenching agent, both the polymers and monomer displayed quenching resulting from the binding of paraquat, while polymer demonstrated greatly enhanced sensitivity compared to the monomer.

![Figure 1-5. Structures of neutral PE monomer (1) and neutral PPEs (2).](image-url)
Optical excitation creates an electron-hole pair, which migrates throughout the polymer (Figure 1-6). The electron transfer quenching occurs when the electron encounters a receptor site occupied with a $\text{PQ}^{2+}$ group. So the polymer needs only a small fraction of receptor sites occupied to effect complete quenching. However, for the monomer, every receptor must be occupied for complete quenching. The degree of sensitivity enhancement is determined by the radiative lifetime and the mobility of the excitations in the polymer. Longer lifetimes and higher mobilities will produce longer average diffusion lengths. If energy migration is rapid with respect to the fluorescence decay, then a single binding site to quencher is able to change the entire emission dramatically. If the diffusion length exceeds the polymer’s length, then an increase in molecular weight will produce greater enhancements.

![Molecular Wire Receptor Assembly](image)

Figure 1-6. Mechanism of amplified quenching by which the molecular wire receptor assembly can produce an enhancement in a fluorescence chemosensory response. Reprinted with permission from Swager et al.9

Swager et al.9 also demonstrated that the energy migration-based amplification is much greater in thin films than in solution. The physical state of the CPE has profound effects on its ability to amplify. In solution, we can presume that the polymers are in random coil form, and the excitons therefore undergo a one-dimensional random walk, which is inefficient for amplification, because the exciton visits the same receptor many times. If the exciton can be
made to undergo vectorial transport in a given direction, then much higher amplification factors can be achieved. Hence, either polymer aggregates or very thin films are preferential for increasing rates of energy transfer in a three-dimensional manner. Close proximity of the neighboring polymer chains facilitates interchain energy migration, and CPEs often exhibit more planar conformations in thin films and aggregates, and these conformations appear to promote exciton diffusion. This concept later led to the development of sensors for nitroaromatics by Swager’s group and commercialization of an explosive detectors, Fido® by ICx Technologies, Inc.\textsuperscript{15}

Another striking discovery of the amplified quenching was reported by Whitten et al.\textsuperscript{16} in 1999 on their study of the fluorescence quenching of MPS-PPV by MV\textsuperscript{2+} (Figure 1-7). This is the first report of the amplified quenching of CPEs, in which the use of an anionic conjugated polymer leads to greater than million-fold amplification of the sensitivity relative to that of corresponding small conjugated molecules with similar structures.

The absorption and fluorescence spectra of MPS-PPV are similar to those studies of \textit{trans}-stilbene and its derivatives, but are shifted to longer wavelength due to the conjugation in polymers. The fluorescence of stilbene analogues can be quenched by electron-deficient MV\textsuperscript{2+} by the formation of “donor-acceptor” complexes.\textsuperscript{17} The quenching efficiency is enhanced when \textit{trans}-stilbene or its amphiphilic derivatives are incorporated into anionic assemblies, such as micelles or bilayer vesicles. This can be attributed to a “concentration enhancement” effect, in which the stilbene and viologen are assembled by a combination of Coulombic and entropic interactions in a microphase, such that their “local” concentrations are greatly enhanced. Remarkably, addition of very low concentrations of MV\textsuperscript{2+} leads to noticeable changes in the absorption spectrum and a dramatic quenching of its fluorescence. The quenching constant (Ksv)
is nearly four orders of magnitude greater than that for stilbene in micelles and six orders of magnitude greater than that for dilute stilbene solutions. Approximately, one molecule of MV$^{2+}$ quenches an entire polymer chain.

By using a combination of steady-state and ultrafast spectroscopy, Whitten et al.\textsuperscript{18} established that the dramatic quenching results from weak complex formation [polymer($^{(-)}$) • quencher($^{(+)})$], followed by ultrafast electron transfer from excitations on the entire polymer chain to the quencher, with a time constant of 650 fs. The ultrafast exciton decay involves two competing quenching mechanisms: aggregation quenching caused by formation of interchain states and electron-transfer quenching caused by the MPS-PPV•MV$^{2+}$ complex. The divalent cation MV$^{2+}$ plays a dual role here: formation of the “donor-acceptor” complex and inducement of polymer aggregation. This is the reason why MV$^{2+}$ is a more effective quencher than Mg$^{2+}$ or Ca$^{2+}$, which can only induce the aggregation of polymers by electrostatic forces, which acts only as an electron acceptor. The amplification of the fluorescence quenching can be further optimized by extending the intrinsic lifetimes of polymer excited states,\textsuperscript{17} and controlling the directional transport of excitons.

Many other groups have also studied the mechanism of fluorescence quenching in CPEs in the following years. It has been shown that the quenching constant (Ksv) can be affected by a
variety of factors, such as polymer concentration, quencher properties, solution properties, and presence of additives.

### Aggregation of CPEs

CPEs are inherently amphiphilic materials and, they tend to aggregate in aqueous solution, even at very low concentrations. In a series of investigations, Schanze et al. have examined the aggregation of PPE and the influence of aggregation on electronic charge carrier transport and energy migration. The photophysics data show that the solvent polarity plays an important role in aggregation of CPEs. As the increasing amount of water, the absorption and fluorescence are red-shifted; the fluorescence spectra and quenched intensity appear as very broad bands. (Figure 1-8) However, in methanol, the absorption and fluorescence spectra are very similar to those of structurally similar, organic-soluble neutral PPEs in ‘good’ solvents, where the aggregation is expected to be minimal. This suggests that in methanol the polymer exists in a non-aggregated state, while in water the polymer is strongly aggregated.

The fluorescence decay time in methanol is dominated by a short-lifetime component with τ =420 ps (amplitude = 97%), while in water the fluorescence decay is biexponential and wavelength dependent, where the fluorescence maximum corresponds to a longer lifetime. The red-shifted absorption band and broad, less efficient, long-lived fluorescence observed from PPE in water suggests an excimer-like state, which is presumably formed via inter-chain interactions. Schanze et al. believe that the increased structural order and conjugation length arise due to face-to-face π-stacking between phenylene rings in adjacent chains. The chains align with their long axes parallel to optimize π-stacking, so that the phenylene rings in each chain should be nearly co-planar. This aggregate conformation reduces the hydrophobic interactions between adjacent polymer chains and, allows the polar sulfonate groups to extend into the aqueous solvent. A larger SV for the fluorescence quenching of PPE is observed in water than in
methanol, suggesting that intrachain exiton migration occurring in the aggregates may lead to further amplification of quenching response.

Bazan et al. studied the solvent-dependent aggregation of a CPE and the influence on energy transfer to chromophore. They reported different aggregation tendencies of water soluble cationic poly [9, 9-bis (6'-N, N, N-trimethylammonium-hexyl) fluorine diiodide] (poly1) in aqueous solutions with varying amounts of THF as well as changes in the fluorescence resonance energy transfer (FRET) of poly1 to dye-labeled DNA (Figure 1-9). Since the backbones and alkyl side chains are hydrophobic moieties, while the cationic charged quaternary amines control electrostatic interactions, the resulting amphiphilic characteristics lead to different aggregation conformations in different solvents. Two aggregation states have been proposed based on the photophysical measurements conducted in the water/THF mixtures. When the THF content is in the range from 30% to 80%, the polymer shows single chain behavior, or weak aggregation. In pure water, the polymers form tight aggregates, which are dominated by the interchain hydrophobic interactions, resulting in lower fluorescence emission intensities due
to π-π interactions. When the THF content is higher than 80%, the ionic interactions of charged groups with the non-polar medium lead to burying of these groups within a new aggregate structure. This aggregation is dominated by the electrostatic interactions of charged quaternary amine groups and iodide counter ions. The aggregation states of polymers influence the contact extent between the polymer and dye-labeled DNA and the distance between the polymers and dyes. So the FRET efficiencies are dependent on the aggregation states.

Figure 1-9. Proposed aggregation modes of cationic polymers in water with different THF content. Reprinted with permission from Wang et al.\textsuperscript{31}

Applications of Conjugated Polyelectrolytes as Sensors

In recent years, the conjugated polymers’ unique optical properties have triggered tremendous exploration of their uses for sensing chemical and biological materials. For example, the conjugated polymers have been effectively employed as sensors to detect metal ions,\textsuperscript{24,25} anions,\textsuperscript{32,33} explosives,\textsuperscript{34,35} small biomolecules,\textsuperscript{36,37} proteins and DNA,\textsuperscript{38,39} etc. Due to the amplification occurred from the conjugated backbones of these polymers, the chemical and biosensors are able to achieve extraordinary sensitivity. Typically, the detection limits for CPE-based biosensors are in the nanomolar range. In a few cases, sensors can even detect the target analytes at the zeptomole level.
The strategy of CPE-based sensors relies not only on the electron transport and energy migration along the conjugated backbone, but also on the conformational changes of polymeric chains. Thus, CPEs can be used to study conformational changes of proteins and DNA at a fundamental level. Based on this platform, CPEs have been employed for a variety of commercial and scientific applications, including the identification of genetic mutations or single-nucleotide polymorphisms (SNPs),\textsuperscript{40} the sensing of amyloid fibril formation,\textsuperscript{40} mercury-sensing based on conformational change of mercury-specific oligonucleotide (MSO),\textsuperscript{41} etc. The CPEs can be fabricated by several methods to meet the needs of different sensing targets: introduction of functional groups to side chains for selectively binding to metal ions or forming helical structures;\textsuperscript{42} attachment of receptors,\textsuperscript{39} peptide linkers or aptamers.\textsuperscript{43} The formats of CPE sensors can be homogenous solution, layer-by-layer assemblies, glass-supported materials and nanoparticle-supported materials. Despite the variety of sensing targets and sensor formats, the underlying sensing strategies are usually divided into three categories: quencher induced quenching-unquenching, chain conformation perturbation, and fluorescence resonance energy transfer.

**Quencher Induced Quenching-Unquenching Mechanism**

Some CPE-based sensors take advantage of the superquenching behavior of CPEs by electron or energy-accepting quenchers. For example, MV$^{2+}$ is an important electronic acceptor quencher for MPS-PPV first reported by Chen and Whitten in 1999.\textsuperscript{17} They constructed a quencher-tether-ligand (QTL) system by covalently linking MV$^{2+}$ via a flexible tether chain to biotin. When this quencher-tether-ligand (QTL) system is mixed with a solution of PPV-$\text{SO}_3^-$, the fluorescence is quenched at very low concentrations of QTL due to the superquenching effect. Addition of small amount of avidin results in recovery of the fluorescence, because the binding of avidin to biotin disrupts the association between polymer and QTL system. As a result, a
fluorescence response is produced when the ligands bind to their specific targets. Using this strategy, Whitten and co-workers have developed a sensor platform based on CPE-coated polystyrene microsphere for detecting enzymatic activity and DNA hybridization.\(^\text{17}\) (Figure 1-10)

![Figure 1-10. Biosensor application based on QTL system. Reprinted with permission from Chen et al.\(^\text{17}\)](image)

Zhao and Schanze discovered that Cu\(^{2+}\) is an efficient quencher of PPE-CO\(_2\)-.\(^\text{27}\) The Stern-Volmer constant (Ksv) for Cu\(^{2+}\) is much higher than that of other metal ions, and is comparable to that of methyl viologen. The strong and selective quenching by Cu\(^{2+}\) likely arises because the metal coordinates with the carboxylate groups of PPE-CO\(_2\)-, and it efficiently quenches the singlet exciton via charge and/or energy-transfer mechanisms. By taking advantage of the efficient quenching of PPE-CO\(_2\)- by Cu\(^{2+}\), a “turn-on” sensor for anions that coordinate with Cu\(^{2+}\) was developed. Some ions with the diphosphate moiety, such as PPI, ATP and ADP, would effectively sequesters the metal ion, disrupts its ability to bind to the carboxylate groups, and recovers the fluorescence intensity of PPE-CO\(_2\)-. This fluorescence response is highly selective to pyrophosphate, compared to other anions including monovalent anions (H\(_2\)PO\(_4\)-, F\(^-\), Cl\(^-\), Br\(^-\), I\(^-\), etc.) and divalent anions (HPO\(_4\)^{2-}, SO\(_4\)^{2-}, etc.). This platform can also be extended to a bio-analytical application by monitoring the activity of alkaline phosphatase (ALP) in real-time. Since ALP catalyzes the hydrolysis of pyrophosphate to monophosphate at physiological pH, the author used PPE-CO\(_2\)-/Cu\(^{2+}\) fluorescent sensor to detect the enzyme’s activity.
Chain Conformation Perturbation Mechanism

CPE-based sensors that transduce the recognition event via the chain conformation perturbation mechanism are widely applied into DNA hybridization detection, protein conformation studies, etc. This strategy usually does not require any chemical reaction of the probes or the analytes. Instead, it is based on different electrostatic interactions and conformational structures. Poly(thiophene) derivatives are commonly used in CPE-sensors of this type because they display chromatic and fluorescent output upon the formation of different conformational structures.

Leclerc pioneered the application of water soluble cationic regioregular poly (thiophene) in the detection of DNA. Poly (3-alkoxy-4-methylthiophene)s were synthesized and formed complexed with single-stranded oligonucleotides or double-stranded (hybridized) nucleic acids. Originally, these polythiophenes are yellow solution with maximum absorption at a short wavelength (Figure 1-11). This corresponds to a random-coil conformation, since any twisting of the conjugated backbone leads to a decrease in the effective conjugation length. Upon adding 1.0 equivalent amount of oligonucleotides, the solution becomes red, because of the formation of a so-called duplex (highly conjugated, planar conformation) between the polythiophene and the oligonucleotide probe. After adding 1.0 equivalent of the perfectly complementary oligonucleotide, the solution returns to yellow, presumably caused by the formation of a new complex termed a triplex (less conjugated, nonplanar conformation), which includes the polymer and the hybridized nucleic acids. For comparison, the single- and two-mismatch oligonucleotide do not form the triplex with polythiophene and the oligonucleic probe, so the solutions stay red when those two are added into duplex solutions. Thus, the sensor is very selective for the oligonucleotide complementary to the probe DNA.
The fluorometric detection of oligonucleotide hybridization is also possible, since the fluorescence of polymer is quenched in the planar, aggregated form as the duplex forms.\textsuperscript{46} When hybridization with the perfect complementary strand takes place, the formation of a polymeric triplex leads to a fivefold increase in fluorescence intensity. By monitoring either the absorption spectrum or fluorescence spectrum, oligonucleotide hybridization can be detected with a high sensitivity ($10^{-14}$ M) and oligonucleotides with one mismatch can be discriminated from the perfect complementary oligonucleotide. Leclerc and coworkers applied a similar strategy to other sensors, including using a cationic polythiophene/DNA based aptamer complex to detect $K^+$ and human thrombin.

In addition, Nilsson and co-workers have a series of publications showing that this mechanism is also effective for the detection of certain protein conformational changes. For example, in 2003, Nilsson and co-workers described how the conformational changes of a synthetic peptide could alter the conformation of an electrostatically bound amino acid-substituted conjugated polyelectrolyte;\textsuperscript{46} and in 2005, Nilsson reported a method to detect
amyloid fibril formation both with a zwitterionic conjugated oligomer and with anionic polythiophene.\textsuperscript{47}

**Fluorescence Resonance Energy Transfer (FRET) Mechanism**

Fluorescence resonance energy transfer is a commonly used signal transduction pathway in biochemical research. Conjugated polymers have the potential to be excellent energy donors in FRET-based sensing schemes. First of all, the high extinction coefficients stemming from their delocalized backbones enable efficient light emission. Second, the excitons generated throughout the entire polymer can migrate to a position on the chain from which FRET is efficient.

![Figure 1-12. The PNA/CPE assay for ss-DNA detection. Reprinted with permission from Liu et al.\textsuperscript{48}](image)

Bazan and Heeger have reported many examples of using CPE to detect specific DNA (or RNA) sequences via FRET to dye-labeled probe molecules.\textsuperscript{44,48} In their systems, the electrostatic attraction between the charged CPE and DNA results in short distances between the donor (CPE) and the acceptor (dye-labeled probe strand). The probe can be PNA, ssDNA, dsDNA or protein (Figure 1-12). The sensing system consists of three parts: the cationic conjugated polyelectrolyte, the probe peptide nucleic acid (PNA) labeled with an energy-accepting chromophore which has strong spectral overlap with the CPE, and the target DNA strand. The PNA is neutral because the phosphate linkages have been replaced with neutral amide linkages, so the average distance
between chromophore and conjugated polymer is too great for efficient FRET. However, when PNA forms stable Watson-Crick base pairs with the complementary single-stranded DNA (ssDNA) target, the resulting complex is strongly negative so it can bind to the conjugated polymer, which allows the efficient FRET to take place. In comparison, noncomplementary ssDNA gives little observable FRET.

Bazan and co-workers also developed a complementary method based on traditional double-stranded DNA (dsDNA) helix formation for the sensing recognition chemistry, instead of the more expensive PNA. In order to alleviate the effects of nonspecific electrostatic interactions between the dye-labeled ssDNA probe strand and the conjugated polymer, Bazan and co-workers used a well-known DNA intercalating dye, ethidium bromide (EB), as an energy acceptor to improve the performance of ssDNA-based assay. Furthermore, they introduced fluorescein as an intermediate “FRET” gate into the ssDNA to allow much more efficient energy transfer to EB, resulting in an 8-fold amplification relative to direct excitation of the intercalator.

Chemiluminescence System

Chemiluminescence (CL) is the light emission as a result of chemical reaction with limited emission of heat. The first CL reaction was prepared by B.Radziszewski in 1877. After that, many more CL reactions were discovered during the early 20th century. Peroxyoxalate chemiluminescence (POCL) is one of the most efficient and versatile CL processes available today. Many studies have been undertaken to determine the mechanisms of the CL reactions. It relies on the reaction between aryl oxalates and an oxidant, typically hydrogen peroxide, to form a high-energy intermediate, where characterization remains controversial. Although the structure of the intermediate is yet to be elucidated, it is believed that it is capable of exciting a large
number of fluorophores.\textsuperscript{52-54} This process, known as sensitization, is independent of irradiation. The chemical reactions in POCL are generally written as follows:

\[
\text{oxalate} + \text{H}_2\text{O}_2 \xrightarrow{\text{base}} \text{high-energy intermediate} \quad (1-1)
\]

\[
\text{high-energy intermediate} + \text{fluorophore} \rightarrow \text{fluorophore}* \quad (1-2)
\]

\[
\text{fluorophore}* \rightarrow \text{fluorophore} + h\nu \quad (1-3)
\]

Commercially available bis(2,4,6-trichlorophenyl)oxalate (TCPO) is widely used in the POCL. Compared to other oxalates, TCPO features relatively higher stability but lower reactivity, so a weak base, generally imidazole (ImH), is employed as the catalyst in the TCPO CL reaction.\textsuperscript{55} POCL is better than other CL systems such as luminal and lucigenin, because the reaction can be carried out at pH 7, the optimal pH for most enzymatic reactions. The sensitivity of POCL is reported to be a 10 to 100-fold improvement over the PL detection method.\textsuperscript{56} The mechanism of POCL reaction is demonstrated in Figure 1-13.

![Figure 1-13. Mechanism of POCL reaction](image)

The main advantage of CL over other techniques is there is no requirement of irradiation of samples with electromagnetic radiation. The absence of a source leads to the elimination of the noise caused by light scattering, background emission and source instability. Because the CL emission can be detected against a much darker background than fluorescence emission, the detection limit can be lower. Meanwhile, the CL reactions are so rapid that usually the maximum
intensity is reached in less than one or two seconds. So CL detection is useful for the rapid on-site analysis, and is suitable for assays which need a large sample throughput in a short period of time. When coupled with flow injection analysis (FIA), liquid chromatography (LC), or capillary electrophoresis (CE), TCPO based POCL systems can be used not only for direct detection of H$_2$O$_2$, fluorophores (e.g. polycyclic aromatic hydrocarbons) or fluorescent derivatized compounds (e.g., amino acids, carboxylic acids and amines), but also for indirect determination of substrates and enzymes (e.g. glucose and glucose oxidase) by detection of H$_2$O$_2$ which is produced through enzymatic reactions. Although the insolubility and hydrolysis of oxalate esters limit the application of POCL systems, aqueous POCL was achieved by combining it with FIA and delivering oxalate solution via a separate flow line.

**CPE-based Real-time Enzymatic Activity Assay**

CPE-based fluorescence assays share the common features of being relatively easy to implement, being highly sensitive and giving a rapid response. In addition, the CPE-based assays are usually carried out in solution under physiological conditions, so that they can provide a real-time signal and also allow determination of enzyme kinetic parameters at very low substrate and enzyme concentrations.

In 2004, Pinto and Schanze used two anionic conjugated polymers, PPESO$_3$ and PPECO$_2$ as the signal-transduction element to develop a turn-on and turn-off sensor, respectively (Figure 1-14). The sensing mechanism relies on an electrostatic interaction between the conjugated polyelectrolyte and a peptide substrate that is labeled with a fluorescence quencher. In the turn-on sensor, the assay is based on the quenching of PPESO$_3$ by two quencher-labeled substrates such as L-Lys-$p$-nitroanilide dihydrobromide (K-pNA) and $N$-benzoyl-Phe-Val-arg-$p$-nitroanilide hydrochloride hydrate (Bz-FVR-pNA), which can be hydrolyzed by thrombin and peptidase, respectively. The enzyme-catalyzed peptide hydrolysis is signaled by an increase in the
fluorescence from the conjugated polyelectrolyte. The turn-on system was used to sense peptidase and thrombin activity for concentrations of enzyme and substrate in the nano-molar regime. Kinetic parameters were recovered from real-time assays.

Figure 1-14. Enzyme kinetics measured by using PPESO3/Bz-FVR-pNA/thrombin assay system. Reprinted from permission with Pinto et al.\textsuperscript{15}

In the turn-off sensor, the PPECO\textsubscript{2} was employed with a “caged” peptide substrate-quencher, \textit{N,N'-bis(carboxybenzylxy-L-arginine amide)}rhodamine-110 dihydrochloride (Rho-Arg-2). This particular derivative is nonfluorescent and does not quench the fluorescence of PPECO\textsubscript{2}. However, when it is hydrolyzed into Rho-Arg, catalyzed by papain, the fluorescence intensity is quenched due to singlet-singlet energy transfer from PPECO\textsubscript{2} to Rho-Arg. The
papain activity can be monitored by a series of assays carried out by using the PPECO₂/Rho-Arg-2/papain system at various concentrations of the Rho-Arg-2 substrate.

Later, Zhao and Schanze developed a real-time turn-off assay to monitor the activity of alkaline phosphatase (ALP). Since ALP catalyzes the hydrolysis of PPI to Pi, the authors can detect the activity of ALP by using PPECO₂/Cu²⁺ system to sense the PPI concentration upon adding different amounts of ALP. Liu and Schanze introduced a sensitive fluorescent turn-off assay for phospholipase C (PLC). The assay is based on the reversible change in fluorescence properties of an anionic CPE (BpPPESO₃) induced by the formation of a polymer-phospholipid complex. The catalytic kinetic parameters, Km and V_max have been determined from the assay.

**Alkaline Phosphatase Assay**

Alkaline Phosphatase (ALP) hydrolyses a wide range of phosphate monoesters in many types of molecules, including nucleotides, proteins, and alkaloids. The assay for ALP enzymatic activity has been the subject of considerable interest owing to the important role that ALP plays in the diagnostic field. Although ALP is present in all tissues throughout the entire body, it is especially concentrated in the liver, kidney, bone and the placenta. Abnormal level of ALP is an important index of several diseases. Elevated ALP level is possibly due to liver dysfunction (hepatitis or liver tumor), bone disease (Paget’s disease, osteosarcoma, osteomalacia, and rickets, etc.), diabetes, bile abduct construction, renal disease and pregnancy. Lower ALP levels may relate to hypophosphatasia, heart surgery, malnutrition, magnesium deficiency, etc.

Among the broad substrates of ALP, pyrophosphate (PPI) has a lower optimum pH compared to other substrates, but the reactivity is relatively low. ALP and PPI are both key regulatory factors in vascular calcification, and they play important roles in clinical calcific vasculopathy and valvulopathy. Hydrolysis of PPI by ALP leads to two free phosphates (Pi). The balance between levels of Pi and PPI controls the formation of hydroxyapatite mineral
crystals and their growth in cartilage and bone. The presence of PPI prevents “soft” tissues from mineralizing, whereas its degradation to Pi catalyzed by ALP facilitates crystal growth. Therefore, development of assays to monitoring the PPI and Pi levels under physiological conditions are essential to the study of cell-mediated phosphate and pyrophosphate metabolism and their effects on regulating calcification.

Many commercialized ALP assay methods have been developed so far. For example, an amperometric analysis method has been designed for indirect measurement of ALP activity using 3-indoxyl phosphate substrate. The hydrolysis catalyzed by ALP gives rise to an indigo product, which is insoluble in aqueous solutions but is easily converted into soluble indigo carmine. This compound is easily detected at a bare screen-printed electrode placed in an Flow Injection Analysis system. In colorimetrical ALP assays, p-nitrophenyl phosphate is usually used as the substrates in alkaline conditions in the presence of phosphate acceptors such as 2-amino-2-methyl-1-propanol. A chemiluminescent immunoassay uses an adamantyl-1, 2-dioxetane phosphate derivative as a substrate for quantification of ALP. The dephosphorylation of the substrate catalyzed by ALP induces light emission with a \( \lambda_{\text{max}} \) of 470 nm. Although these methods are highly sensitive, none of them have used PPI as the substrate. Therefore Liu and Schanze developed a fluorescent assay using PPI as the substrate, and this assay enables the continuous detection of PPI concentration (Figure 1-15).

This sensor is based on the amplified quenching of PPE-CO\(_2\)\(^-\) by Cu\(^{2+}\) and the binding between PPI and Cu\(^{2+}\). The fluorescence of PPE-CO\(_2\)\(^-\) is initially quenched by Cu\(^{2+}\). Then the addition of PPI to the PPE-CO\(_2\)\(^-\)/Cu\(^{2+}\) solution causes the fluorescence to recover by disrupting the polymer-metal complex. Upon hydrolysis of PPI into Pi catalyzed by ALP, the fluorescence is quenched again, since the Pi is unable to complex with Cu\(^{2+}\). By monitoring the concentration
of PPI as a function of enzymatic reaction time, which is calibrated with fluorescence intensity, the kinetics of ALP activity can be investigated and Pi inhibition can also be studied.

Figure 1-15. Mechanism of fluorescent ALP assay. Reprinted with permission from Liu et al.73
CHAPTER 2
ALKALINE PHOSPHATASE SENSORS USING CHEMILUMINESCENT CONJUGATED POLYMER

Introduction

Although the photoluminescence (PL) of CPE has been investigated in depth, the chemiluminescence (CL) of CPE has never been studied to our best knowledge. The POCL system achieves superior sensitivity and detection limits compared to PL detection methods. However, POCL-based assays for biological analytes can be applied only to those which involve H$_2$O$_2$ in enzymatic reactions. While CPEs are employed as fluorophores in POCL systems, the biosensor potential of CPEs make it possible to expand CL application to biological targets which are not related to H$_2$O$_2$. The combination of the amplified quenching effects of CPE with the intrinsic high sensitivity of CL is expected to improve the properties of possible biosensors.

In previous work in the Schanze research group, Liu developed the first CL system for CPE utilizing TCPO as CL reagent, H$_2$O$_2$ as oxidant, ImH as catalyst and an anionic CPE (BpPPESO$_3$) as fluorophore in aqueous/acetonitrile (CH$_3$CN) solvent mixtures. The results showed that the luminescence of BpPPESO$_3$ is not influenced by the presence of H$_2$O$_2$, ImH or TCPO in the solution, an essential prerequisite for CPE-based CL system. The CL intensity vs. wavelength profile is identical to the PL spectrum of BpPPESO$_3$ in the same solvents with maximum emission at 450 nm. The CL intensity vs. time profile showed that the intensity rises sharply to the maximum value within 5 s and decays gradually until falling to almost zero after about 30 s.

The optimum reagent concentrations and solvent composition were determined by evaluating their effects on CL signals, taking into consideration both maximum intensity and signal duration. Figure 2-1a demonstrates the effects of [H$_2$O$_2$] on the signal, with 375 µM BpPPESO$_3$, 1.5 mM ImH and 0.5mM TCPO in 50/50 THF/H$_2$O solvent system. The CL

37
intensity increases linearly with [H$_2$O$_2$] and reaches a plateau at about 30 mM H$_2$O$_2$. The CL duration time increases with [H$_2$O$_2$] at low concentrations until it reaches its maximum at 4 mM H$_2$O$_2$, a moderate excess relative to the oxalate. Therefore, 8 mM H$_2$O$_2$ was chosen for subsequent experiments as a compromise of the CL intensity and duration time.

Figure 2-1. Optimization of CL signals ($\lambda_{em} = 450$ nm). (a) Effect of H$_2$O$_2$ concentration on CL intensity and duration time. (b) Effect of ImH concentration on CL intensity and duration time. (c) Effect of solvent composition on CL intensity and duration time. These experiments were carried out by Liu in the Schanze research group.

The effect of [ImH] on CL signal is illustrated in Figure 2-1b. The CL reaction conditions were 375 µM BpPPESO$_3$, 1.5mM H$_2$O$_2$ and 0.5 mM TCPO in 50/50 THF/H$_2$O. The CL intensity and duration time increase to their maximum when [ImH] is 2 mM and 1mM, respectively. However, they both decrease as [ImH] increases from 2mM to 25 mM. This is probably due to the decrease of CL quantum yield with excess ImH, from the perspective of kinetics of CL reaction. Another possibility is that excess ImH results in the breakdown of TCPO to generate
1,1’-Oxalylidimidazole (ODI) which is confirmed to be the main precursor formed in the TCPO-CL reaction. Finally, 1.5 mM was selected as the concentration of ImH after compromising between CL intensity and duration time.

Regarding effects of [TCPO], CL intensity increases almost linearly with increasing of initial [TCPO] from 0 to 1.5 mM in the presence of 375 µM BpPPESO₃, 8mM H₂O₂ and 1.5 mM ImH. However, CL duration time is independent of [TCPO], because the POCL reaction is pseudo-first order when H₂O₂ is in moderate excess compared to TCPO. In this case, [TCPO] has no effect on the rate constant of the POCL reaction. Since TCPO has limited solubility in H₂O, TCPO is dissolved in CH₃CN and then mixed with other reactants in water solution to initiate the CL reaction.

Effects of water contents on the CL signals have also been investigated. Figure 2-1c shows the CL vs. time profiles with 10 µM BpPPESO₃ using different volume fractions of H₂O in H₂O/CH₃CN mixtures, decreasing from 80% to 20%. There is no reasonable CL signal when more than 75% of H₂O is present, because TCPO hydrolyses rapidly before inducing CL reaction at such high water content. The CL intensity increases with decreasing of water content and then remains almost unchanged from 50% to 20% H₂O. The reasons may be the increased stability of TCPO in the less aqueous environment, as well as increased quantum yield of BpPPESO₃ in the organic solvent. The CL duration time also increases with decreasing water content, which may be due to the increase in rate of the based-catalyzed TCPO reaction at high water concentrations or decreased consumption of TCPO from hydrolysis. However, in order to develop a chemiluminescence-based assay for enzyme, a higher water content is preferred to mimic the physiological conditions. Therefore, 50% water content was chosen for the CL-based assays that will be described later.
Results and Discussion

Photophysical Property of PPECO₂

PPECO₂ is an anionic CPE having carboxylate side groups (Figure 2-2a). The synthesis and characterization of this polymer is included in the early literatures.²⁷ Similar to other CPEs, PPECO₂ shows solvent-dependent absorption and emission properties. In an organic solvent (methanol), PPECO₂ exhibits an absorption maximum at 417 nm and an emission maximum at 437 nm. With increasing water amount, the polymer shows a red-shift and narrowing absorption spectrum, as well as a significant red-shift and broadening of the fluorescence spectrum. In pure water, it absorbs at maximum of 435 nm and exhibits an emission maximum at 520 nm.

The solvent-dependent photophysical properties of CPEs are due to the aggregation of the polymer in aqueous solution. In methanol, the absorption and emission spectra of this polymer correspond well with the spectra of structurally analogous polymers that contain alkyl or alkoxy-solubilizing groups in good solvents. In water, the spectra display spectral changes that are characteristic of aggregate formation as seen in the solid films of the structurally similar organic polymers.

![Figure 2-2. Structures of PPECO₂ (a) and TCPO (b).](image)

Chemiluminescence of PPECO₂

The chemiluminescent reaction based on PPECO₂ is not affected by the presence of ImH, H₂O₂ and TCPO, which is the prerequisite for this CL system. Since TCPO hydrolyzed easily in
water, it is dissolved in CH$_3$CN first, and then mix with the buffer solution containing PPECO$_2$, ImH, and H$_2$O$_2$ to initiate the CL reaction. The CL intensity is recorded instantaneously when the TCPO/CH$_3$CN solution is added into the aqueous mixture. The CL intensity vs. time profile of 25 µM PPECO$_2$ is measured at the emission wavelength of 435 nm. The optimum conditions for the CL system has been investigated by Liu and, the final concentrations are set to be 25µM PPECO$_2$, 8 mM H$_2$O$_2$, 1.5 mM ImH and 0.3 mM TCPO in H$_2$O/CH$_3$CN (50/50) solvent system.

![Figure 2-3](image)

Figure 2-3. CL intensity of PPE-CO$_2^-$. (a) The photos of CL system (right) and control system (left). Condition: 25 µM PPE-CO$_2^-$, 8 mM H$_2$O$_2$, 1.5 mM ImH, 0.3 mM TCPO (no TCPO in the control system) in Tris-HCl/CH$_3$CN (50/50) solvent. (b) The CL intensity in different buffer/CH$_3$CN system. Conditions: 25 µM PPE-CO$_2^-$, 8 mM H$_2$O$_2$, 1.5 mM ImH, 0.3 mM TCPO in Tris-HCl/CH$_3$CN (50/50) ( ), H$_2$O/CH$_3$CN (50/50) ( ), HEPEs/CH$_3$CN (50/50) ( ), $\lambda_{em} = 446$ nm.

The CL profile is characterized by two processes: the intensity rises sharply to the maximum within 5 seconds, and then decays gradually until falling to nearly zero. The rise is due to the formation of high-energy intermediate as well as excitation of fluorophore. The decay is related to the loss of TCPO in the CL reaction. The CL measurement shows good reproducibility upon repeating the tests. The CL intensity of PPECO$_2$ is strong enough to be observable in the dark, compared to the control which has no TCPO added (Figure 2-3a).
In order to utilize this CL system into biosensor, the buffer effect on CL signal needs to be studied. The CL intensities of PPECO₂ were measured in different buffers/CH₃CN (50/50) mixtures (Figure 2-3b). It turns out that the CL intensity of PPECO₂ is around three times higher in Tris-HCl than that in Hepes, while two times higher than that in water. The reason for that is not fully understood, but it might results from stabilizing TCPO by Tris-HCl.

**Quenching Studies**

The amplified quenching of CPE by small molecules with opposite charges has been extensively studied, either in aqueous or organic solvents. The mechanism is attributed to a combination of ion-pairing between CPE and quencher with energy and exciton rapid migration and/or delocalization within the polymer chain to the quencher binding site. Therefore, quenching study is the most convenient method to evaluate the CPE-based TCPO-CL system. Cu²⁺ has been reported to be a quencher for PPECO₂ and is a requirement in ALP assay application. The quenching experiment was carried out by a series of Cu²⁺ titration of the CL reaction system in Tris-HCl buffer/CH₃CN solvent (including 25 µM PPECO₂, 8mM H₂O₂, 1.5 mM ImH and 0.3 mM TCPO). As a comparison, the PL quenching experiment was conducted in the same solvent mixture with the same concentration of each reactant except for the absence of TCPO.

Figure 2-4A and Figure 2-4B illustrates the quenching of PPECO₂ by Cu²⁺ in the PL and in the CL system, respectively. The difference is for PL quenching, emission vs. wavelength profiles are used; for CL quenching, emission vs. time profiles are used. The results of Stern-Volmer plots for the quenching of PPECO₂ in PL and CL system have been illustrated in Figure 2-4C. The integrated areas of CL/PL profiles are used as signal intensities. The Ksv for the PL and CL system are 1.9 × 10⁵ and 4.5 × 10⁵ M⁻¹, respectively. The quenching efficiency of CL is higher than that of PL in the same solvent mixture.
Figure 2-4. A) Fluorescence intensity and B) chemiluminescence intensity of PPE-CO$_2^-$ upon titration of Cu$^{2+}$. Fluorescence: 25 µM PPE-CO$_2^-$ in Tris-HCl (10 mM, PH 7.5) /CH$_3$CN (50/50) with 8 mM H$_2$O$_2$, 1.5 mM ImH, 0.3 mM TCPO, $\lambda_{ex} = 380$ nm. Chemiluminescence: 25 µM PPE-CO$_2^-$ in Tris-HCl (10 mM, PH 7.5)/CH$_3$CN (50/50) with 8 mM H$_2$O$_2$, 1.5 mM ImH, $\lambda_{em} = 446$ nm. (c) Stern-Volmer plots of fluorescence and fluorescence quenching of PPE-CO$_2^-$ by Cu$^{2+}$. 
Although PPECO$_2$ shows amplified quenching property beyond the linear range, the CL/PL quenching efficiency in the mixture of aqueous and CH$_3$CN is about 10-fold smaller compared with a typical “superlinear” PL quenching as seen in water solution with no H$_2$O$_2$, ImH or TCPO added ($K_{sv} \sim 10^6$ M$^{-1}$). Two factors account for the less efficiency of quenching: first, the presence of organic solvent, CH$_3$CN, induces less aggregation of CPE, less ion-pairing forces and less hydrophobic interaction between CPE and small charge quencher; second, the presence of ImH, H$_2$O$_2$ or TCPO may change the polymer condition or solution environment. However, the quenching of CL still demonstrated the amplified quenching property of CPE with about 10 to 100 times larger than the quenching efficiency of the small model compound.

**Alkaline Phosphatase Chemiluminescence Turn-off Assay**

A real-time fluorescence turn-off assay for ALP utilizing PPECO$_2$ has been developed in the previous work of our group. The same approach was ready to be implemented by means of TCPO-CL. Figure 2-5 shows the mechanism of the CL turn-off assay for ALP.

![Figure 2-5. Mechanism of CL turn-off assay for ALP.](image)

The CL of PPECO$_2$ is quenched efficiently by Cu$^{2+}$ via charge and/or energy transfer mechanism. The quenched CL is recovered upon the addition of inorganic PPI, which is due to
the strong association of PPI with Cu$^{2+}$, thus disrupting of PPECO$_2$/Cu$^{2+}$ complex. Introduction of ALP to the mixture of PPECO$_2$/Cu$^{2+}$/PPI initiates the hydrolysis of PPI to Pi, which is lack of ability to complex with Cu$^{2+}$. As the reaction proceeds, the amount of PPI to associate with Cu$^{2+}$ decreases. As a result, the CL of PPECO$_2$ is quenched by the free Cu$^{2+}$ again. Therefore, the presence and activity of ALP is detected by the decline of CL intensity.

Figure 2-6. Chemiluminescence intensity quenched by Cu$^{2+}$ and then recovered by PPI.

The CL quenching experiment of PPECO$_2$ by Cu$^{2+}$ was discussed in the section of quenching studies. Typically, with mixture of Tris-HCl (10mM, pH 7.5)/CH$_3$CN (50/50) containing 8 mM H$_2$O$_2$, 1.5 mM ImH, 0.3 mM TCPO, the initial CL intensity of 25uM PPECO$_2$ is completely quenched by 50 µM Cu$^{2+}$. The quenched CL was recovered to 80% of initial intensity upon addition of 75 µM PPI; (Figure 2-6) and the recovery curve was level off after this amount. Introduction of ALP into the Tris-HCl solution containing PPECO$_2$/H$_2$O$_2$/ImH/Cu$^{2+}$/PPI and incubation of it for some time allows the hydrolysis of PPI by ALP. It would result in the decrease of CL intensity generated by mixing Tris-HCl solution with TCPO CH$_3$CN solution. The above concentrations of each species were chosen as the condition of CL turn-off assay for
ALP. The emission wavelength to record the CL time profiles was set at 446 nm, because it is the maximum emission wavelength achieved from PL spectrum of PPECO\textsubscript{2} in Tris-HCl/CH\textsubscript{3}CN (50/50).

![Figure 2-7A](image1.png)

**Figure 2-7A.** Changes of chemiluminescence intensity observed in the turn-off assay for ALP. A) Changes of chemiluminescence intensity after 5 min of addition of different concentrations of ALP. B) Linear calibration plot of chemiluminescence intensity decrease as a function of ALP concentration. Conditions: 25 µM PPE-CO\textsubscript{2} -, 8 mM H\textsubscript{2}O\textsubscript{2}, 1.5 mM ImH, 0.3 mM TCPO, 50 µM Cu\textsuperscript{2+} and 75 µM PPI in Tris-HCl (10 mM, PH 7.5) /CH\textsubscript{3}CN (50/50), \(\lambda_{em} = 446\) nm, at 37 ºC.

Figure 2-7A illustrates CL spectroscopic changes observed with 5 min of incubation of different concentration of ALP with substrate in the turn-off assay at 37 ºC. It shows clearly that the CL intensity decreases with increase of initial ALP concentration in a rage of 100 – 2000 nM. Figure 2-7B displays a linear correlation between intensity change and amount of ALP added,
where \( I_0 \) and \( I \) are initial and decreased CL intensities upon addition of ALP; the integrated area of CL profile was used as CL intensity. It suggests that it should be possible to quantitatively determine the level of ALP by this CL assay.

Figure 2-8 shows the decrease of CL intensity in the presence of 400 nM ALP as a function of incubation time. The initial CL intensity of PPECO\(_2\) which was displayed as 0 min of assay was normalized to 1. The relative CL intensity keeps decreasing with incubation time, approaching about 15% of initial intensity at 30 min of incubation and rarely changing after this.
It is demonstrated that most PPi is hydrolyzed and enzymatic reaction almost completes in the first half of hour.

**Discussion**

Compared with PL turn-off assay for ALP with detection limit of ~ 5.0 nM ALP within one minute of response, the CL ALP assay is less sensitive. In combination with above quenching study as well as CL turn-on assay for peptidase, the detection sensitivity of TCPO-CL system of CPE is not as good as that of PL system. The necessary of organic solvent, CH₃CN, to dissolve TCPO is one of the main reasons to account for this problem. It induces the decrease the quenching efficiency of the polymer and the change of the buffer condition which is important to enzyme activity. TCPO hydrolyses to a more or less extent in aqueous environment, which reduces its ability as CL reagent.

Therefore, to find a substitute for TCPO with better water solubility and stability as CL reagent is advantageous to eliminate the need of organic solvent. An oxamide reagent with two sulfonate groups on the benzene rings, which is specially designed for aqueous CL reagent, is a good example as the substitute. Another reason for the low sensitivity may arise from the mixing method used in the CL process. Manually pipette-mixing is simple but less efficient. The approach to solve this problem and improve the performance of the CL system is to couple it with flow injection or stop-flow which provides rapid and reproducible mixing, thus giving efficient CL monitoring, and allows rapid sample throughput.

By improving the sensitivity of CL assay utilizing these two methods, plus its inherent merit of simple and direct measurement, TCPO-CL approach opens a new path for luminescence CPEs in biosensor application.
Experimental

Chemicals

PPE-CO$_2^-$ (Figure 2-2) was synthesized according to literature methods and concentrated aqueous solution of them were diluted with water or buffer solution to a final concentration ranging from 10 to 25 µM. All chemicals were used as received, unless otherwise noted. Bis(2,4,6-trichlorophenyl) oxalate (TCPO) and imidazole (ImH) were purchased from Acros. 1,1’-Oxalyldiimidazole (ODI) was obtained from Sigma-Aldrich. Hydrogen peroxide (H$_2$O$_2$), 30% solution in water was purchased from Fisher Chemical. The concentration of H$_2$O$_2$ was determined by titration with the solution of potassium permanganate prior to dilution. HPLC-grade acetonitrile (CH$_3$CN) was obtained from Fisher and used without further purification. Water was distilled and then purified by using a Millipore purification system. Stock solutions of 2.0 mM TCPO in CH$_3$CN and 10.0 mM ImH in H$_2$O were prepared immediately before their use. TCPO solution was stored in dark prior to dilution and analysis.

The quencher, copper (II) chloride (Cu$^{2+}$) was obtained from Sigma-Aldrich. Stock solutions of Cu$^{2+}$ in water were prepared before the quenching test and adjusted to 1.0 mM. The substrate for alkaline phosphatase, sodium pyrophosphate (PPi), was purchased from J. T. Baker Chemical Company. The enzyme, alkaline phosphatase bovine intestinal mucosa (ALP) was purchased from Sigma-Aldrich. Buffer solution was prepared with reagent-grade material from Fisher. Stock solutions of enzymes in the appropriate buffers were prepared immediately before their use in the fluorescence assays. Prior to ALP CL assay, PPi and ALP were dissolved in Tris buffer solution (Tris-HCl, 10 mM, pH 7.5) and adjusted to 2.0 mM and 20 µM as stock solution. The ALP assay was carried out in the same buffer solution.
Chemiluminescence Measurements

In a typical CL measurement, an aliquot of aqueous solution containing 10-25 µM PPECO₂, 10 – 40 mM H₂O₂ and 1.875 – 7.5 mM ImH was pipetted into the cuvette which was placed in spectrometer. An aliquot of 0.375 mM – 1.5 mM TCPO CH₃CN solution was pipetted into above solution in dark to initiate the CL reaction. The emitted light was detected simultaneously. The profile of CL intensity vs. wavelength was recorded on a home-built spectrometer without switching on the excitation source. The spectrometer was equipped with a Triax 180 spectrograph (ISA-Spex) with liquid N₂ cooled silicon CCD detector (EEV CCD chip, 1024 × 128 pixels). The profile of CL intensity vs. time was measured at a specific wavelength on a JOBIN YVON-SPEX Industries Fluorolog-3 spectrofluorometer (Model FL3-21) with the lamp turned off. The emission wavelength to record time profiles was chosen from maximum wavelength in the PL spectrum measured under the same condition but with the lamp turned on. The CL signal (intensity and duration time) were acquired as a function of the concentration of each reagent (PPECO₂, H₂O₂, ImH and TCPO) and ratio of solvents (H₂O/CH₃CN). The CL intensity is the peak intensity in the CL vs. time profile, and the CL duration time refers to the range of time from addition of TCPO to the moment when the intensity does not fall anymore. The concentration of each reagent shown in the results and discussion section is the calculated concentration after mixing different ratio of aqueous and CH₃CN solutions.

Quenching Behavior

The CL quenching test was done by a series of titration of PPECO₂/H₂O₂/ImH aqueous solution by Cu²⁺. It was followed by addition of TCPO CH₃CN solution and measurement of CL vs. time profile. The integrated area of profile, which is proportional to CL intensity, was used as intensity of CL signal here. The PL quenching study in water was conducted in the same manner without addition of TCPO.
Alkaline Phosphatase Chemiluminescence Assay with PPECO₂

The ALP assay was carried out at 37 °C. The initial CL intensity of PPECO₂ was recorded first. The Cu²⁺ solution was then added to another PPECO₂/H₂O₂/ImH mixture, the solution was incubated for 10 min, and the quenched CL intensity was measured. Subsequently the PPi solution was introduced to another freshly prepared PPECO₂/H₂O₂/ImH/Cu²⁺ solution, this mixture was incubated for 10 min, and the recovered CL intensity was recorded. Finally, an aliquot of ALP solution was added to another fresh PPECO₂/H₂O₂/ImH/Cu²⁺/PPi solution, and the CL profiles were measured as a functions of incubation time and concentration of ALP. The incubation time refers to the period after introduction of ALP into the aqueous solution but before mixing with TCPO CH₃CN solution to initiate CL reaction. The integrated area of profile was used as intensity of CL signal.
CHAPTER 3
ALKALINE PHOSPHATASE SENSORS USING FLUORESCENT DENDRITIC POLYMER

Introduction

Dendritic polymers form a special class of macromolecules composed of molecular chains that branched out from a common center. There are four major subclasses of dendritic polymers: random hyperbranched polymeric architectures: dendrigraft polymers, dendrons, and dendrimers. Usually there is no entanglement between dendrimer molecules. The unique physical and chemical properties of these materials has led to a wide range of applications: adhesives and coatings, chemical sensors, medical diagnostics, drug-delivery systems, high-performance polymers, catalysts, building blocks of supermolecules, separation agents and etc.

Figure 3-1. Four major subclasses of dendritic polymers. (a) Random hyperbranched. (b) Dendrigrafts. (c) Dendrons. (d) Dendrimers. Reprinted with permission from Tomalia et al."74

This chapter focuses on the dendritic CPEs, which belong to dendrigrafts subclasses and their application in biosensors. Since the CPEs tend to aggregate in good solvents such as water, addition of dendritic side-groups to the conjugated backbone helps keep the molecules apart, leading to significantly enhanced luminescence quantum efficiencies. Some research shows that the dendritic side-group architecture may accommodate a variety of moieties that could perhaps enhance transport properties and alter the mechanical characteristics of the polymer. In dendritic CPEs, the conjugated backbone defines the key electronic properties such as the absorption and emission wavelength, while the surface groups control processing properties, such as solubility.
The disruption of CPE aggregates in aqueous solution by introduction of dendritic groups has been reported in the literatures. Due to the influence of intermolecular interactions on both charge transport and light emission, the molecular engineering of conjugated dendrimers allows control of the spacing of polymer assemblies and can be used to make highly efficient solution-processed LEDs.

In the Schanze research group, Zhao recently synthesized PPE-\textsuperscript{d}NH\textsubscript{3}Cl (Figure 3-2) and examined its optical properties under varying conditions, such as solvent, pH, and ionic strength. As mentioned above, the polymer’s optical properties are mainly determined by the structure of the conjugated backbone. Compared to similar conjugated polymers carrying linear side-groups, the absorption maxima of dendritic polymers are blue-shifted, due to the more twisted backbone conformation caused by the increased electronic repulsion between the charged dendritic groups. The maximum fluorescence emission wavelength for PPE-\textsuperscript{d}NH\textsubscript{3}Cl has negligible shift compared to its linear counterpart.

![Figure 3-2. The structure of PPE-\textsuperscript{d}NH\textsubscript{3}Cl.](image)

Zhao also checked the solvent effects on absorption and fluorescence of this dendritic polymer by varying the MeOH/H\textsubscript{2}O ratios in the solvent mixtures. The absorption spectra showed little change, while the fluorescence decreased in intensity. But compared to their linear counterparts, their fluorescence is still relatively efficient and retains high quantum efficiency in
water. The results suggest that the interchain aggregation of dendritic CPEs in the aqueous solutions is attenuated by attaching the bulky ionic groups.

Figure 3-3. Absorption (a) and fluorescence (b) spectra of PPE-dNH₃Cl in water as a function of pH. [PPE-dNH₃Cl] = 5µM, pH range from 4.5 to 10.5 in 1.0 pH unit intervals. These experiments were carried out by Xiaoyong Zhao in the Schanze group.

Figure 3-3 shows the absorption and emission spectra of PPE-dNH₃Cl in water as a function of pH. When the solution is acidic, the neighboring phenylene ethynylene groups are twisted in the polymer to minimize the electrostatic repulsion between the positively charged ammonium side groups. With increasing pH, the ammonium groups are deprotonated, which leads to the planarization of the conjugated backbone. The fluorescence of PPE-dNH₃Cl is quenched with increasing pH, which indicates that aggregation of PPE-dNH₃Cl is occurring. Meanwhile, the red-shifted, broad band dominating the fluorescence spectrum is believed to arise from the π-π stacking of the polymer chains.

The interactions between cationic dendritic conjugated polymers and small negative ions including PPi and Pi were also investigated. Figure 3-4 shows the absorption and fluorescence spectra of PPE-dNH₃Cl upon titration of PPi and Pi. As PPi is added, the absorption maximum is red-shifted concomitant with the emergence of a new low-energy band, and the fluorescence intensity is quenched. It is believed that PPi induces aggregation of polymer chains by
neutralizing the positive charges on the ammonium side groups. However, when the negative Pi is added, negligible effects are observed.

![Figure 3-4](image)

Figure 3-4. The absorption (a) and fluorescence (b) spectra of PPE-\(^{d}\)NH\(_3\)Cl upon titration of PPi. The absorption (c) and fluorescence (d) spectra of PPE-\(^{d}\)NH\(_3\)Cl upon titration of Pi. 

By taking advantage of the distinct fluorescent response of PPE-\(^{d}\)NH\(_3\)Cl with addition of PPi and Pi, we developed a fluorescent turn-on assay for ALP using cationic dendritic CPE. The enzymatic assay employs PPE-\(^{d}\)NH\(_3\)Cl as an amplified fluorescent transducer to monitor the PPi activity of alkaline phosphatase under physiological conditions in real-time. The new ALP assay based on the quenching-unquenching mechanism is more convenient and sensitive than the former assays for alkaline phosphatase.

**Results and Discussion**

**Overview of Alkaline Phosphatase/PPi assay**

Although a sensitive real-time ALP assay has previously been developed in our group,\(^{27}\) the method requires Cu\(^{2+}\) as a signal transducer between CPE and the substrate, since there is no
direct correlation between the fluorophore and substrate. The fluorescence of CPE is first quenched by \( \text{Cu}^{2+} \) via a charge-transfer mechanism, and the fluorescence is subsequently recovered by PPi, since PPi sequesters the \( \text{Cu}^{2+} \) by complexation. In order to derive a relationship between fluorescence intensity and PPi concentration, the concentration of \( \text{Cu}^{2+} \) has to be established. An arbitrary logarithmic calibration plot is employed to convert the fluorescence signal into substrate concentration, which eventually introduces errors and affects the precision of the assay method.

In this chapter, we describe a real-time ALP assay using PPE-\(^4\text{NH}_3\text{Cl}\), which displays a direct fluorescent response to PPi but no fluorescent response to Pi. Since PPi is a substrate for ALP, which catalyzes the hydrolysis of PPi to Pi, this property of PPE-\(^4\text{NH}_3\text{Cl}\) provides a unique platform for a turn-on assay via a quenching-unquenching mechanism. The mechanism of this assay is illustrated in Figure 3-5. The PPE-\(^4\text{NH}_3\text{Cl}\) features dendritic ammonium side groups, which induce three-dimensional separation of the conjugated backbone and enhances the luminescence quantum efficiency. The random-coil conformation dominates the photophysics of the polymer. With the addition of PPi, the repulsive interaction between neighboring polymer chains is reduced by neutralizing the positive charges in the sidegroups. Furthermore, pyrophosphate may complex with the dendritic sidegroups from different polymer chains, which brings the neighboring polymer chains into close proximity and promotes the planarization of polymer aggregates. So the fluorescence of PPE-\(^4\text{NH}_3\text{Cl}\) is quenched by addition of a small amount of PPi. Then, ALP is added to the polymer solutions. ALP catalyzes the hydrolysis of PPi to Pi, which exists in the form of \( \text{HPO}_4^{2-} \) and \( \text{H}_2\text{PO}_4^- \) in the buffered solution (pH 7.5). Pi is unable to induce aggregation of PPE-\(^4\text{NH}_3\text{Cl}\), so the polymer assemblies are disrupted and the fluorescence is recovered. As catalyses proceeds, the amount of PPi available to complex with
dendritic sidegroups decreases, and thus the fluorescence intensity of PPE-$d$NH$_3$Cl increases with time. With a calibration plot of fluorescence of PPE-$d$NH$_3$Cl versus PPi concentration, the amount of substrate PPi can be monitored by time-resolved measurement of the fluorescence. This allows determination of the kinetic parameters and inhibition studies of ALP activity.

![Figure 3-5. Mechanism of ALP “turn-on” sensor using PPE-$d$NH$_3$Cl.](image)

In preliminary research, MES (10 mM, pH 6.5) was used as the buffer system to study the photophysical properties of PPE-$d$NH$_3$Cl. As shown in Figure 3-4, the optical properties of PPE-$d$NH$_3$Cl are strongly affected by pH in aqueous solution. In acidic solution, the bulky side groups in PPE-$d$NH$_3$Cl are positively charged, which promotes the three-dimensional separation of polymer chains by electrophobic repulsion. However, with increasing pH, the ammonium groups are deprotonated which reduces the repulsive forces between neighboring chains so the polymers tend to aggregate. Therefore, the absorption is red-shifted and the fluorescence is quenched. In order to apply PPE-$d$NH$_3$Cl in an ALP assay, two conditions are prerequisite. First, the initial conformation of polymers has to be random-coil to maintain relatively high fluorescence intensity before it is quenched by PPi. Second, the assay has to be usable under physiological conditions. The pyrophosphatase activity has been reported to show an upward trend with the increase of pH up to 9.2. In order to compromise these two requirements, MES buffer at pH 6.5 is used. At pH 6.5, the polymers retain high quantum yields, and the fluorescence spectra
correspond to non-aggregated conformation. But the pyrophosphatase activity of ALP is not affected adversely.

**Quenching Studies of PPE-d\textsuperscript{d}NH\textsubscript{3}Cl by PPi**

According to previous research conducted by our group, PPE-d\textsuperscript{d}NH\textsubscript{3}Cl can be selectively quenched by negative ions with charges more than three, including ATP, ADP and PPi, while it cannot be quenched by those negative ions charges lower than three, including AMP and Pi. Similar results have been obtained for the polythiophene derivative, which has been applied in the detection of ATP.\textsuperscript{76} As described above, the PPE-d\textsuperscript{d}NH\textsubscript{3}Cl by itself is in a random-coil conformation with twisted conjugated backbone due to the electron repulsion caused by the positively charged bulky side groups. The negative charges in ATP, ADP or PPi promote the formation of a $\pi$-stacked supramolecular complex, the higher the negative charge in the molecule, the more fluorescence quenching can be achieved for the same concentration. The adenosine in the biological molecules such as ATP and ADP may enhance the $\pi$-stacked aggregates of polymer chains. So the fluorescence quenching mechanism is suggested to be anion-induced aggregation of cationic conjugated polymers.

Addition of PPi ($c = 0 – 10 \ \mu\text{M}$) into the 10 $\mu\text{M}$ PPE-d\textsuperscript{d}NH\textsubscript{3}Cl solution in MES buffer (10 mM, pH 6.5) results in the quenching of fluorescence (Figure 3-6). The ratio between initial fluorescence ($I_0$) and quenched fluorescence ($I_q$) as a function of quencher concentration ([PPi]) affords the Stern-Volmer plot, which displays a superlinear shape (Figure 3-6B).

Similar to other CPE/quencher systems, the plot is linear at lower quencher concentration, but it curves upward at higher quencher concentrations. The Stern-Volmer constant (Ksv) can be derived from the linear region of the superlinear correlation as $2.7 \times 10^5 \ \text{M}^{-1}$. In order to carry out kinetic studies of pyrophosphatase activity of ALP, a calibration plot for determination of [PPi] from the fluorescence intensity is needed. The linear region of the Stern-Volmer plot (up to
4 mM PPi), affords a correlation coefficient $R^2 = 0.9886$, and the intercept is very close to the theoretical value of 1.00, indicating a reasonable fit to derive the kinetic parameters. The following equation is derived from the Stern-Volmer equation and can be used to calculate PPi concentrations from the fluorescence intensities directly:

$$[Q]_t = [Q]_0 \times \frac{I_0 - 1}{I_0}$$

(2-1)

![Figure 3-6](image)

Figure 3-6. (a) The fluorescence changes upon titration of PPi into a solution of 10 µM PPE-$^d$NH$_3$Cl in 10 mM MES buffer (pH 6.5) at 37 °C, $\lambda_{ex} = 380$ nm. (b) Stern-Volmer plot of fluorescence quenching of PPE-$^d$NH$_3$Cl by PPi. Inset: Calibration curve for the fluorescence quenching by PPi.

In Equation 2-1, $[Q]_0$ and $[Q]_t$ represent the initial quencher concentration and the quencher concentration during the enzymatic reaction at time t, respectively; $I_0$ represents the fluorescence...
intensity before the quencher is added; \( I_Q \) represents the fluorescence intensity quenched by initial concentration of quencher before the addition of enzyme; \( I_t \) represents the fluorescence intensity during the enzymatic reaction at time \( t \).

**Real Time ALP Turn-on Assay**

To guarantee the feasibility of this ALP assay, a series of control experiments were performed to examine the effect of the chemicals involved in the assay on the fluorescence of PPE-\(^{d}\)NH\(_3\)Cl (Figure 3-4). The hydrolysis product, phosphate, has negligible effect on the fluorescence of PPE-\(^{d}\)NH\(_3\)Cl. The enzyme ALP also has no effect on the fluorescence of PPE-\(^{d}\)NH\(_3\)Cl. These control experiments rule out the possibility that the fluorescence intensity changes arise from any interference by these components in the solution.

![Figure 3-7. Fluorescence changes observed in the ALP turn-on assay. Increase of fluorescence intensity at 430 nm recorded every 10 sec during the real-time ALP turn-on assay with varying concentrations of ALP. Conditions: [PPE-\(^{d}\)NH\(_3\)Cl] = 10 µM, [PPI] = 20 µM in the MES buffer (10 mM, pH 6.5) at 37 °C, \( \lambda_{ex} = 380 \) nm, \( \lambda_{em} = 431 \) nm.](image)

In practice, the ALP turn-on assay is conducted by first measure the initial fluorescence of 10 µM PPE-\(^{d}\)NH\(_3\)Cl to obtain \( I_0 \). Then the fluorescence is quenched using 20 µM PPI, and the quenched fluorescence intensity, \( I_Q \), is measured. After addition of ALP solution, the fluorescence keeps rising with the hydrolysis of PPI, since the polymer aggregates are
dissembled in the absence of PPI. The fluorescence intensity, $I_t$, can be monitored by time-resolved measurement, which affords the enzymatic reaction processing curve (Figure 3-7). The hydrolysis rate increases with increasing enzyme concentration. Since the fluorescence intensity increases linearly with time when the enzyme concentrations are in the range of 10-100 nM, which means the rate of reaction is constant over the time range of the measurement for these concentrations.

Figure 3-8. Decrease of [PPI] during the enzymatic reaction in the ALP turn-on assay with varying ALP concentrations. Conditions: [PPE-$^4$NH$_3$Cl] = 10 µM, [PPI]$_0$ = 4 µM, in MES buffer (10 mM, pH 6.5), at 37 °C, $\lambda_{ex} = 380$ nm, $\lambda_{em} = 431$ nm.

To study the kinetics of ALP hydrolysis by this assay, the initial reaction rate is determined by first converting the fluorescence intensity, $I_t$, into substrate concentrations using the calibration plot. Since the calibration curve is linear only when the PPI concentrations are below 4 µM, the initial substrate concentration is set to be 4 µM in the kinetic experiments. Then the processing curve can be transformed into the plot of PPI concentration as a function of time using Equation 2-1. The plots in Figure 3-8 are linear in the first 60 seconds. The slopes of these lines afford the values of the initial velocity of the enzymatic reaction with different ALP concentrations.
Kinetic Studies of ALP Turn-on Assay for Pyrophosphatase Activities

To derive the kinetic parameters from the processing curves above, the plots of fluorescence changes as a function of time are converted to the plots Ln [PPi] vs. time for five different enzyme concentrations ranging from 50 nM to 400 nM, when the substrate concentration is kept at a constant value 4 µM (Figure 3-9). The kinetic parameter, $V_{\text{max}}/K_{M}$ can be calculated from the slopes of the plots. (See Experimental part) These $V_{\text{max}}/K_{M}$ values are plotted as a function of ALP concentrations, which affords a linear relationship, because $V_{\text{max}}$ should be directly proportional to enzyme concentration (Figure 3-9 inset). The slope of this linear plot affords a value of enzyme efficiency, $k_{\text{cat}}/K_{M} = 2.18 \times 10^{4}$ M$^{-1}$s$^{-1}$, which is in reasonable agreement with the $k_{\text{cat}}/K_{M}$ value ($3.13 \times 10^{3}$ M$^{-1}$s$^{-1}$) obtained by Liu et al. using a PPE-CO$_2$/Cu$^{2+}$/PPi sensor. The observed $k_{\text{cat}}/K_{M}$ is also within the range of $k_{\text{cat}}/K_{M}$ ($1.6 \times 10^{2}$ ~ $8.9 \times 10^{4}$ M$^{-1}$s$^{-1}$) obtained from a different ALP assay systems in a previous literature.77

This experiment shows the PPE-$^4$NH$_3$Cl/PPi assay is able to detect the pyrophosphatase activity of ALP in the nanomolar range of enzyme concentration. The assay allows kinetic studies by monitoring the rapid fluorescence responses. The detection limit of ALP enzyme calculated from the plot of $V_{\text{max}}/K_{M}$ vs. [ALP] is about 29 nM. However, the detection limit can be even lower when higher substrate concentration is used. (Figure 3-7)

Additionally, a series of assays were conducted to monitor the hydrolysis of pyrophosphate with different initial substrate concentrations, in which the ALP concentration was maintained at 100 nM. The initial substrate concentrations were varied over the range of 1 µM - 4 µM. Figure 3-10 shows plots of the changes of product concentration [Pi] (obtained by difference, [PPi]$_0$ to [PPi]$_t$) as a function of reaction time with different initial substrate concentrations. Although the substrate concentrations are low and the concentration range is narrow, the fluorescence response curve in different samples can still be resolved within 200 seconds. The slopes of these plots
increase with substrate concentrations as expected, because the initial substrate concentrations are far below the $K_m$ values reported in the previous literatures.\cite{68,78-81}

Figure 3-9. Natural logarithm of the concentration of substrate for ALP activity as a function of reaction time plotted for different enzyme concentrations. Inset: $V_{\text{max}}/K_m$ as a function of enzyme concentration. Conditions: $[\text{PPE-dNH}_3\text{Cl}] = 10 \mu M$, $[\text{PPi}]_0 = 4 \mu M$, in MES buffer (10 mM, pH 6.5), at $37 \, ^\circ\text{C}$, $\lambda_{\text{ex}} = 380 \, \text{nm}$, $\lambda_{\text{em}} = 431 \, \text{nm}$.

Figure 3-10. Concentration of the hydrolysis product Pi as a function of time at various initial substrate concentrations in ALP catalyzed reaction system. Conditions: $[\text{PPE-dNH}_3\text{Cl}] = 10 \mu M$, $[\text{ALP}] = 100 \, \text{nM}$, in MES buffer (10 mM, pH 6.5), at $37 \, ^\circ\text{C}$.
Inhibition Studies of the ALP Activity

The PPE-dNH₃Cl/PPi system was also used to investigate the inhibition of the ALP activity. Since inorganic phosphate has been reported to be an inhibitor of pyrophosphatase activity,⁸²-⁸⁴ phosphate can be added into the enzyme systems to reduce the hydrolysis rate of the PPi. Results demonstrate that the initial reaction rate decreases with the rising of phosphate concentrations.

![Dixon plot](image)

Figure 3-11. Inhibition of ALP activity by inorganic phosphate. Conditions: [PPE-dNH₃Cl] = 10 µM, [ALP] = 100 nM, in MES buffer (10 mM, pH 6.5), at 37 °C, $\lambda_{ex} = 380$ nm, $\lambda_{em} = 431$ nm, with the presence of two substrate concentrations: 3 µM PPi (black dot) and 4 µM PPi (red dot). The data represented in the Dixon plot, displaying a $1/v_0$ vs. [I] dependence for each [PPi].

To derive the inhibition constant $K_i$ in this assay, a series of phosphate samples with increasing concentrations were added to the ALP assay systems with constant ALP concentration. The reciprocal of initial reaction rate was plotted as a function of phosphate concentration at two different initial substrate concentrations. (Figure 3-11) These two plots, called Dixon plots, correspond to two different substrate concentrations, respectively; converge in the left upper quadrant. This proves phosphate is a competitive inhibitor of pyrophosphatase activity of ALP, which means phosphate competitively binds to the same active site of ALP as pyrophosphate. The intersection point of these affords a value of $K_i$ to be 4.67 µM, which is comparable to the
value obtained in a former ALP assay using \( p \)-nitrophenylphosphate as substrate (5.3 \( \mu \)M). This means the pyrophosphatase activity in this ALP assay system is very sensitive to phosphate inhibition. Therefore, we suggest that the pyrophosphates activity is inhibited by the product formation. This explains the plots of fluorescence response vs. time are initially linear, but they level off with the extended incubation time, because of the build-up of product Pi.

**Selectivity of PPE-DNH\textsubscript{3}Cl/PPi Turn-on Assay for ALP**

The selectivity of the PPE-DNH\textsubscript{3}Cl/PPi assay for ALP activity was evaluated by comparing the fluorescence responses to ALP to those with other proteins (Peptidase, BSA, Peroxidase, Glucose oxidase, Phospholipase, and hexokinase). Since none of these proteins has a specific interaction with PPi, no fluorescence response is expected to be observed from these systems.

![Figure 3-12](image-url)

Figure 3-12. The fluorescence responses of PPE-DNH\textsubscript{3}Cl/PPi (10 \( \mu \)M/20 \( \mu \)M) to various proteins with concentration of 200 nM in MES buffer (10 mM, pH 6.5), at 37 °C. \( \lambda_{ex} = 380 \) nm, \( \lambda_{em} = 431 \) nm.

The PPE-DNH\textsubscript{3}Cl/PPi (10 \( \mu \)M/20 \( \mu \)M) mixture in MES buffer (10 mM, pH 6.5) has low fluorescence intensity due to the quenching of fluorescence of PPE-DNH\textsubscript{3}Cl by PPi. The proteins (200 nM) listed above were added to PPE-DNH\textsubscript{3}Cl/PPi mixtures, and then incubated at 37 °C for 60 minutes. The ratios of fluorescence intensity after incubation to that before incubation are
shown in Figure 3-12. As expected, only ALP is able to turn-on the fluorescence intensity, while the other proteins induce only small fluorescence changes, which may arise from nonspecific interactions. The fluorescent response is 20–90 fold larger in PPE-\(^{d}\)NH\(_3\)Cl/PPi/ALP system than in other protein systems. This shows that the PPE-\(^{d}\)NH\(_3\)Cl/PPi is highly selective to ALP because of the specific pyrophosphatase activity of ALP.

Discussion

This ALP turn-on assay for pyrophosphatase activity is based on the amplified quenching of the fluorescence of PPE-\(^{d}\)NH\(_3\)Cl by pyrophosphate. The distinct fluorescent responses of PPE-\(^{d}\)NH\(_3\)Cl to pyrophosphate and phosphate allow us to monitor the hydrolysis of pyrophosphate to phosphate catalyzed by ALP.

Although this is not the first ALP assay using a conjugated polyelectrolyte, this assay is superior to the former ALP turn-off assay using PPE-CO\(_2\)^{−}, which was also developed by our group. First, in the new assay, the quencher of the fluorophore is also the substrate of ALP enzyme. This highly sensitive sensor relies on the sensitive fluorescent response of the PPE-\(^{d}\)NH\(_3\)Cl to the perturbation from small amount pyrophosphate. Therefore, only the substrate, enzyme and the fluorophore are involved in the new assay. This makes this assay easier and more efficient because fewer reagents are needed, which means the assay is more amenable to being applied in a high-throughput screening (HTS) technology. Second, the Stern-Volmer equation can be directly used as a calibration plot to convert the fluorescence intensity into substrate concentrations. This simplifies the assay by eliminating the cumbersome calibration steps. This not only makes the kinetic studies more convenient but also avoids the errors introduced in the calibration process. Third, the PPE-\(^{d}\)NH\(_3\)Cl/PPi system is simpler than the previous assay. The chances of inducing interference from nonspecific interactions are reduced to some extent. This greatly improves the selectivity of this assay to ALP over other proteins,
with 20~90 fold increase of intensity. The fluorescence of dendritic cationic PPE-\textsuperscript{d}NH\textsubscript{3}Cl also shows amplified quenching response to other biological anions including ATP and ADP, while no response to AMP. The distinct fluorescence responses of PPE-\textsuperscript{d}NH\textsubscript{3}Cl to different anions provide a unique platform to develop more assays for other enzymes which use these anions as substrates. This ALP turn-on assay paves the way for broader application of CPEs into enzymatic activities assays.

Although there are many advantages of this ALP turn-on assay, some limitations are still yet to be addressed. The Stern-Volmer plot is only linear at low substrate concentrations, so the calibration plot is valid only over a narrow PPi concentration range (0-4 µM). The sensitivity of this assay is undoubtedly affected by this limitation because fewer reaction processing plots can be collected in order to guarantee the resolution. Also, because the fluorescence intensity of PPE-\textsuperscript{d}NH\textsubscript{3}Cl is sensitive to pH, the assay is performed at pH 6.5, which is not the optimum pH for enzyme activity. In order to improve the ALP assay, more CPEs being designed to maintain high fluorescence quantum yields at higher pHs.

**Experimental**

**Materials**

All stock solutions were prepared with water that was distilled and then purified by using a Millipore purification system. PPE-\textsuperscript{d}NH\textsubscript{3}Cl was synthesized by Zhao Xiaoyong in our group. MES buffer solution was prepared with reagent-grade material from Fisher. The concentrated solution of PPE-\textsuperscript{d}NH\textsubscript{3}Cl is diluted with MES buffer solution to a final concentration of 10 µM. All chemicals were used as received, unless otherwise noted. Sodium pyrophosphate (Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}) was purchased from J.T.Baker Chemical Company, and sodium phosphate tribasic (Na\textsubscript{3}PO\textsubscript{4}) was obtained from Fisher Scientific. Each reagent was dissolved in water and adjusted to 50 mM as stock solution. Alkaline Phosphatase from bovine intestinal mucosa (ALP) and the other control
proteins including peptidase from porcine intestinal mucosa (PTD), bovine serum albumin (BSA), peroxidase from horseradish Type I (HRP), glucose oxidase from Aspergillus niger (GOx), phospholipase A$_2$ from bovine pancreas (PLA$_2$) and hexokinase (HK) were purchased from Sigma-Aldrich. These proteins were dissolved in MES buffer and adjusted to 20 µM as stock solution. The enzyme solutions were freshly prepared immediately before their use in assays. All the assays were conducted in the same buffer solution.

**Instrumentation**

Fluorescence spectra were recorded on a spectrofluorometer from Photon Technology International and corrected by using correction factors generated with a primary standard lamp. The 1-cm fluorescence cuvette was placed in a custom-built thermostatted cell holder which was maintained at 37 °C during the assay and was equipped with a micro-submersible magnetic stirrer.

**Fluorescence Assays**

The enzyme assays were carried out in MES buffer (10 mM, pH 6.5) at 37 °C. All the fluorescent intensities were recorded with excitation wavelength of 380 nm and emission wavelength of 431 nm. A 2-ml aliquot of polymer solution was placed in a cuvette, and the initial fluorescence intensity ($I_0$) was measured after the sample was allowed to equilibrate thermally. The substrate was then added, and after incubating for 10 min, the fluorescence intensity ($I_Q$) was again measured. For end-point assays, an aliquot of the enzyme solution or protein solution was then added and then incubated for 60 min, the fluorescence intensity was recorded. For real-time assays, after the PPE-$d^4$NH$_3$Cl/PPi solution was incubated for 10 min, the fluorescence intensity was first recorded at 10-s intervals as a blank ($I_{bl}$). The fluorescence intensity decreased with time due to the photobleaching of polymer. Then another freshly prepared PPE-$d^4$NH$_3$Cl/PPi solution was incubated for 10 min, an aliquot of enzyme solution was
added to the mixture, instantly the fluorescence intensity was recorded at 10-s intervals ($I_t$). The signal $I_t$ was corrected by $I_{bt}$ for photobleaching effect.

**Calculation of Kinetic Parameters and the Inhibition Constants**

The corrected fluorescence intensities, were converted to substrate concentrations $[Q]_t$ using Equation 2-1. The real-time fluorescence plots were then transformed into enzymatic reaction processing curves. The $[PPi]_t$ vs time plots were initially linear and then leveled off. The initial velocities were calculated from the slopes of the linear regions in the plots.

The Michaelis-Menten equation is usually used to derive kinetic parameters and expressed as

$$v_0 = \frac{V_{\text{max}} \times [S]_0}{K_m + [S]_0} \quad (2-2)$$

where $v_0$ is initial rate of reaction, $[S]_0$ is the initial substrate concentration, $V_{\text{max}}$ is the maximum rate of reaction, $K_m$ is the Michaelis-Menten constant. In this assay, the initial substrate concentrations are far below the reported $K_m$ values. So this equation can be transformed into

$$v_0 = \frac{V_{\text{max}} \times [E]_0}{K_m} \left( \frac{[E]_0 [S]_0}{K_m} \right) \rightarrow V_{\text{max}} = \frac{K_{\text{cat}} [E]_0}{K_m} \quad (2-3)$$

where $[E]_0$ is the initial enzyme concentration, $k_{\text{cat}}$ is catalytic constant or turnover number, and $k_{\text{cat}}/K_m$ is called as specificity constant. According to Equation 2-3, the specificity constant $k_{\text{cat}}/K_m$ can be obtained from the slope of a plot $V_{\text{max}}/K_m$ vs. $[E]_0$. Meanwhile, the initial velocity can be expressed by Equation 2-4:

$$v = \frac{d[PPi]_t}{dt} = \frac{V_{\text{max}} [E]_0}{K_m} \quad \rightarrow \frac{d[PPi]_t}{dt} = \frac{V_{\text{max}} [E]_0}{K_m} \quad \rightarrow \frac{d(\ln[Q]_t)}{dt} = \frac{V_{\text{max}}}{K_m} \quad \frac{dE}{dt}$$

We can infer that $V_{\text{max}}/K_m$ can be calculated from the slope of the natural logarithm of $[PPi]_t$ vs time plot. So from the natural logarithm of $[PPi]_t$ vs time plots at different initial enzyme concentrations, we derived the $V_{\text{max}}/K_m$ for different enzyme concentrations, which affords a linear plot of $V_{\text{max}}/K_m$ vs. $[E]_0$. From the slope of this plot, we derived the specificity constant $k_{\text{cat}}/K_m$ for ALP enzyme.
To study the competitive inhibition of ALP activity by phosphate, the inhibition constant of ALP activity $K_i$ was calculated by Dixon plots. The reaction velocity was measured at a fixed concentration (3 µM or 4 µM) of substrate but at a variety of inhibitor concentrations ranging from 0-10 µM. Two plots of the reciprocal of velocity against inhibitor concentrations are plotted using two different substrate concentrations. A vertical line from the intersection point of these two plots to the inhibitor axis gives -$K_i$. 
CHAPTER 4
CONCLUSION

In the previous chapters, the development and optimization of two optical biosensors for alkaline phosphatase activity using functionalized poly(para-phenylene ethynylene) (PPE) have been presented. Both of the ALP assays are based on the quenching-unquenching mechanism, which takes advantage of the amplified fluorescence responses of conjugated polyelectrolytes (CPE) to small molecular quenchers. Therefore, they feature both good sensitivity and specificity for the enzymatic activity assay. The ALP sensor described in Chapter 2 uses an imidazole-catalyzed peroxyoxalate chemiluminescence reaction to excite the fluorophore poly(phenylene ethynylene) carboxylate (PPE-CO$_2^-$). This method displays the advantages of simple instrumentation with no need for a light source. Chapter 3 described a fluorescent ALP assay using poly(phenylene ethynylene) with charged amino dendritic sidegroups (PPE-$^{4}$NH$_3^+$) to monitor the rapid fluorescence responses caused by the hydrolysis of pyrophosphate in real time. These results allowed the derivation of kinetic parameters and inhibition studies.

**Chemiluminescent Conjugated Polyelectrolyte**

The chemiluminescence system is based on imidazole-catalyzed peroxyoxalate chemiluminescence reaction which consists of bis(2,4,6-trichlorophenyl) oxalate (TCPO) as CL reagent, H$_2$O$_2$ as the oxalate, ImH as the catalyst and PPE-CO$_2^-$ as fluorophore in buffer/CH$_3$CN(50/50) solvent. The chemiluminescence of PPE-CO$_2^-$ shows an amplified quenching response to Cu$^{2+}$, because Cu$^{2+}$ induces the aggregation of conjugated backbones. The sensitivity of the chemiluminescent quenching of PPE-CO$_2^-$ is even higher than that of fluorescent quenching of the same polymer under the same conditions. The quenched chemiluminescence of PPE-CO$_2^-$ can be recovered by PPI since PPI sequesters Cu$^{2+}$ from the polymer aggregates by forming a complex with Cu$^{2+}$. Upon addition of ALP into the PPE-CO$_2^-$
/Cu\textsuperscript{2+}/PPi system, the chemiluminescence intensity is quenched again, because ALP hydrolyzes PPi into Pi, which is not able to complex with Cu\textsuperscript{2+}. Therefore, the Cu\textsuperscript{2+} is released from the complex with PPi and forms aggregates with polymer chains, resulting in decreased chemiluminescence intensity with incubation time. Also, the ratio of initial chemiluminescence intensity from PPE-CO\textsubscript{2} over the quenched chemiluminescence intensity displays a linear relationship with the concentration of ALP concentration. This means the chemiluminescent conjugated polyelectrolyte provides a platform for quantitative analysis of ALP enzyme.

This CL-based endpoint sensor using CPE utilizes simple in instrumentation while maintaining good sensitivity. However, the chemiluminescence intensity of conjugated polyelectrolyte is sensitive to the solvent system, being highest in Tris•HCl/CH\textsubscript{3}CN, followed by water/CH\textsubscript{3}CN and then HEPEs/CH\textsubscript{3}CN. CH\textsubscript{3}CN is a necessity in the system in order to stabilize the CL reagent. To extend the application of chemiluminescent conjugated polyelectrolytes into enzymatic sensors, we hope to improve the CL system by using more water stable chemiluminescent reagents and introducing more efficient sampling technology such as flow injection.

**Fluorescent Real-Time ALP Assay**

The PPE-\textsuperscript{d}NH\textsubscript{3}Cl-based turn-on assay for pyrophosphatase activity of ALP is based on the direct interaction between the cationic dendritic polymer PPE-\textsuperscript{d}NH\textsubscript{3}Cl and small negative PPi ions. The dendritic amino protonated side groups in PPE-\textsuperscript{d}NH\textsubscript{3}Cl promote the three-dimensional separation of the neighboring polymer chains by electrophobic repulsions. Thus, the PPE-\textsuperscript{d}NH\textsubscript{3}Cl is in random-coil conformation with high quantum yield. However, the fluorescence of PPE-\textsuperscript{d}NH\textsubscript{3}Cl is highly sensitive to small amounts of anions with more negative charges such as ATP, ADP and PPi, while insensitive to those with less negative charges such as AMP and Pi. The PPi is believed to form aggregates with PPE-\textsuperscript{d}NH\textsubscript{3}Cl inducing the planarization of the
polymer backbones by complexation with the amino dendritic groups and resulting neutralization of the positive charges from the side groups. Therefore, the fluorescence intensity of PPE-$^d$NH$_3$Cl is strongly quenched by PPi, affording a superlinear Stern-Volmer plot. The quencher PPi is also the substrate of the ALP for pyrophosphatase activity. So the hydrolysis of PPi into Pi catalyzed by ALP can be monitored by recording the fluorescent responses of PPE-$^d$NH$_3$Cl/PPi upon addition of ALP in real time. Since the linear range of the Stern-Volmer plot can be used to convert the fluorescence intensity into the PPi concentrations, the time-resolved fluorescence spectra are conveniently transformed into enzymatic reaction processing curves. These plots allow the derivation of kinetic parameters and the competitive inhibition by phosphate.

This real-time turn-on ALP assay using cationic dendritic CPE is easy to perform, convenient for kinetic studies, highly selective to ALP, free of non-specific interference and amenable to High-throughput screening (HTS) technology as well as biological applications. Also, the ALP assay can be extended to other enzymatic assays, because PPE-$^d$NH$_3$Cl shows distinct responses to other biological anions, such as ATP, ADP and AMP. However, several limitations are yet to be addressed: first, the narrow substrate concentration ranges affect the sensitivity of the assay; and second, the fluorescence of PPE-$^d$NH$_3$Cl is sensitive to pH. The optical properties of dendritic CPEs are expected to be improved to further advance their applications to biological sensors.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Lijuan Huang was born in 1983, in Jiangxi province, China, and she spent her childhood and youth there. In 1999, she left her hometown and began her undergraduate studies in Wuhan University in Hubei province, China, and obtained her B.S. degree in chemistry in 2003. She continued her study in Shanghai Institute of Organic Chemistry, Chinese Academia of Sciences and was awarded the M.S. degree in biochemistry in 2006. After that, she was accepted by the Chemistry Department at the University of Florida (Gainesville) and joined the Schanze group for research on biosensors using conjugated polymers until now.