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Recent advances in fluorescent and colorimetric conjugated polymer-based biosensors

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Conjugated polymers recently have drawn much attention as an emerging sensory material due to their meritorious signal amplification, convenient optical detection, readily tunable properties, and easy fabrication. We review the molecular design principles of sensory conjugated polymer recognition events, which can trigger conformational change of the conjugated polymer, induce intermolecular aggregation, or change the distance between the conjugated polymer as an energy donor and the reporter dye molecule as an energy acceptor. These recognition/detection mechanisms result in mainly three types of measurable signal generation: turn on or turn off fluorescence, or change in either visible color or fluorescence emission color of the conjugated polymer. In this article, we highlight recent advances in fluorescent and colorimetric conjugated polymer-based biosensors.

Introduction

The medical diagnostics and biomedical research fields rely on research that enhances the sensitivity and selectivity of biosensor technology. Researchers have pursued optical biosensors that use various modalities due to their quick and simple functionality and visual signaling. Conjugated polymers (CPs) have recently received worldwide attention as effective optical transducers. π-CPs are compounds with alternating saturated and unsaturated bonds and backbone atoms that are sp^1 or sp^2-hybridized.[1] This p-orbital overlap provides conductivity in chemically doped CPs and fluorescence in CPs that undergo radiative energy transfer when light is absorbed. Changes in the chemical nature, effective conjugation length, intramolecular conformation and intermolecular packing can change the absorption and fluorescence properties of CPs, thus providing mechanisms that can be implemented in sensing applications.[2–13] By incorporating specific functional moieties that can bind to molecules of interest, CP biosensors have been developed that either have a change in fluorescence or a change in color when the molecules of interest are present.

As a sensing platform, CPs are more advantageous than small molecule-based sensors because they are able to amplify the signal from a recognition event.[14–17] When an analyte binds locally to a functional receptor on a CP, the backbone and electronic properties of the entire CP are affected. The recognition event

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results in amplification or quenching of fluorescence when compared to small molecule sensors because a binding event on a small molecule only causes a single chromophore to change its fluorescence, whereas a CP binding event affects the fluorescence of an entire chain of macromolecular chromophores. This signal amplification provided by CPs is important for biosensing applications because the molecules being analyzed are often present in extremely dilute concentrations.

Several detection modes have been developed for the sensing of biomolecules by CPs including fluorescence turn-on (enhancing) and turn-off (quenching), fluorescence color change and visible color change (Fig. 1). When the turn-on mechanism is implemented, target binding perturbs the electron density along the CP backbone or changes the conformation of the polymer chain, which results in CP fluorescence. In a turn-off system, a binding event can cause the fluorescence of a CP to be effectively quenched through non-radiative relaxation pathways in a chain or through the intermolecular aggregation of polymer chains.[11,18–22] Fluorescence color change may arise after a target binding event from a change in electron density along the conjugated backbone of a CP. The driving force of the CP charge density change may be polymer aggregation,[11,19–21,23–30] conformational change of the CP backbone,[31–42] and electron energy transfer.[21,24,25,27,36,43–48] Furthermore, for any of the fluorescent modes the fluorescent signal can be amplified using fluorescence resonance energy transfer (FRET).[49] This is an energy transfer mechanism between two chromophores that has been used to transfer energy from a CP to a reporting fluorophore or quencher. Visible colorimetric sensors are also convenient and useful clinically since the signal can be seen by the "naked eye".[34] This mode is triggered when a binding event causes a change in the conjugation length of a CP backbone and thus changes the wavelengths of light absorbed by the polymer.

Both fluorescent and colorimetric CP biosensors have been developed with specific biological receptors to detect DNA, proteins, and various small biological molecules. This Highlight will review the recent progress in bio-/synthetic CPs as fluorescent and colorimetric transducers to increase the sensitivity and selectivity of biomolecular detection. Specific emphasis will be placed upon detection types and the mechanisms that induce amplified sensitivity.

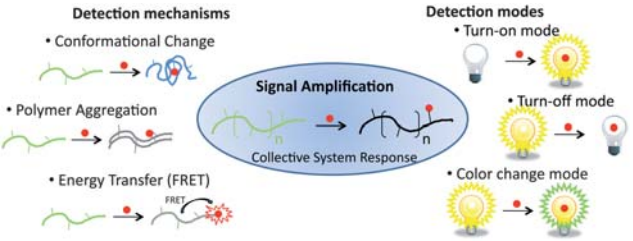

Fig. 1 Detection mechanisms and modes for CP biosensors.

Fluorescent conjugated polymer biosensors

Solution-state sensors

The motivation for developing aqueous solution-based CP sensors is derived from the state of clinically interesting target molecules. Since many biological targets in biosensors require an aqueous environment to maintain their form and function, it is often necessary to have water-soluble target receptors and reporters. Furthermore, CP water-solubility is a prerequisite for in vivo sensors and imaging applications. To meet this requirement, water-soluble and highly emissive conjugated polyelectrolytes (CPEs) have become emerging candidates for solution-state sensors. Poly(phenylenevinylene)-based CPEs were introduced by Shi and Wudl in 1990[50] and have been further developed by Whitten and coworkers.[21] Additionally, the concept of signal amplification by CPs for sensory applications was proposed by Zhou and Swager in 1995[51] and the amplified fluorescence quenching mechanism of poly(*p*-phenyleneethynylenes) (PPE)-based CPEs was further proposed by Schanze.[10,11] As highlighted in these articles, other polymers have been developed in recent years for biosensor applications. However, since CPs have carbon-based hydrophobic and rigid-rod like backbones by nature and easily aggregate in aqueous environments, it is essential to introduce hydrophilic side chains and ionic pendant groups such as sulfonate (SO^{3-}), carboxylate (CO^{2-}), phosphonate (PO_4^{3-}), and quaternary ammonium (NR^{3+}). These pendant groups, often combined with poly(ethylene oxide) side chains, help CPEs dissolve in water and prevent the aggregation of multiple chains. Recent reviews published by several researchers give a more in-depth overview of the potential applications of new CPE structures, including those with poly(*p*-phenylene), poly(phenylene vinylene) and poly(phenyleneethynylene) backbone structures.[52–55]

The driving force behind CPE amplified fluorescence quenching or enhancement comes from aggregation or conformational change of CPEs[18,21,42,56–60] or a change in physical proximity from quenching molecules.[10,11,59,61–63] Bunz and coworkers reported fluorescence self-quenching of poly(p-phenyleneethynylene) (PPE) derivatives that were modified with carbohydrate chains after polymerization. The *turn-off type* PPE shows fluorescence quenching upon the addition of Concanavalin A (Con A), the lectin protein, through the formation of non-fluorescent aggregates among multiple PPE chains and Con A.[22] In a similar

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manner, other sugar-substituted PPE derivatives have been reported for Con A and *E. Coli* detection.[64] Also, Liu *et al.* recently showed that intermolecular aggregation can be used to induce fluorescence color change in water-soluble poly(benzothiadiazole-fluorene) copolymer derivatives upon the addition of protein target.[18] They have further developed copolymers containing benzothiadiazole units for detection of heparin.[65] The aggregation concept was recently used to develop a fluorescence turn-on sensor with high selectivity using DNA-mimetic poly(*p*-phenylenebutadiynylene). The sensor is based on the intermolecular packing change of the polymer backbone, which is functionalized with thymine (T) nucleobases. Initially, the polymers fluorescence is quenched due to aggregation. When DNA with adenine (A) nucleobases is added the fluorescence of the polymer is revived.[66]

FRET-based fluorescence color change mode, which incorporates another dye or a quencher molecule, is popular for CP solution sensors. CPs have high extinction coefficients and effective one-dimensional migration of excitons throughout their conjugated backbones. This enables an exciton to move to a position on the polymer chain from which FRET can happen efficiently. The efficiency of FRET in CPEs is strongly influenced not only by their quantum yield but also by the charge density along the polymer backbone.[67] Therefore, the design of polymer molecules used in FRET-based sensing should be carefully considered in order to favor FRET. Fig. 2 schematically illustrates signal amplification mechanism by FRET when a rationally designed CPE-DNA recognizes its target complementary DNA.

CPEs have the potential to be excellent energy donors for FRET-based DNA detection in aqueous media. Bazan and Heeger developed a solution-based DNA sensor using water-soluble cationic conjugated poly(fluorene-*co*-phenylene)s in 2002.[23] The strategy uses fluorescein-labeled peptide nucleic acid (PNA), which can form stable Watson–Crick base pairs with a target single-stranded DNA (ssDNA) for sensing. When the PNA is mixed with cationic CPs the average distance between the polymer and the PNA is greater than the effective FRET distance because there is no electrostatic attraction between them. However, once negatively charged complementary DNA is introduced to the solution, DNA hybridizes with PNA and the DNA/PNA complex electrostatically binds to the positively charged polymer. This brings the polymer and the dye attached to PNA close enough for FRET to happen. The authors also reported the use of a conventional DNA/DNA duplex sensor based on the previous results for the PNA/DNA sensor. Instead of using PNA, dye-tagged probe ssDNA strands were used.[24] When the polymer and dye-labeled DNA are incubated with target complement a stable polymer/DNA/DNA triplex is formed due to DNA/DNA hybridization. In this conformation FRET from the polymer to the dye amplifies the fluorescent signal. Wang and co-workers also developed single nucleotide polymorphism (SNP) sensors using the same concept.[68] The target used was the mutant DNA fragment Arg282Trp, which replaces the nucleotide guanine (G) in the wild-type fragment with the nucleotide adenine (A). The 3′terminal of the DNA probe contains the nucleotide thymine (T) and thus is complementary to the mutant-type target sequence and is not complementary to the wild-type target. The probe was extended using Taq DNA polymerase and fluorescein labeled-dGTP as the G base. Therefore, the mutant target duplex has fluorescein at the end of 3′-terminus of the probe while the wild-type target does not. By adding a blue-emissive CPE as an energy donor, fluorescein, which accepts energy from the polymer, emits an amplified signal only when the mutant target is present.

The sensitivity and selectivity of CP DNA sensors are limited by non-specific binding between dye-labeled anionic ssDNA and

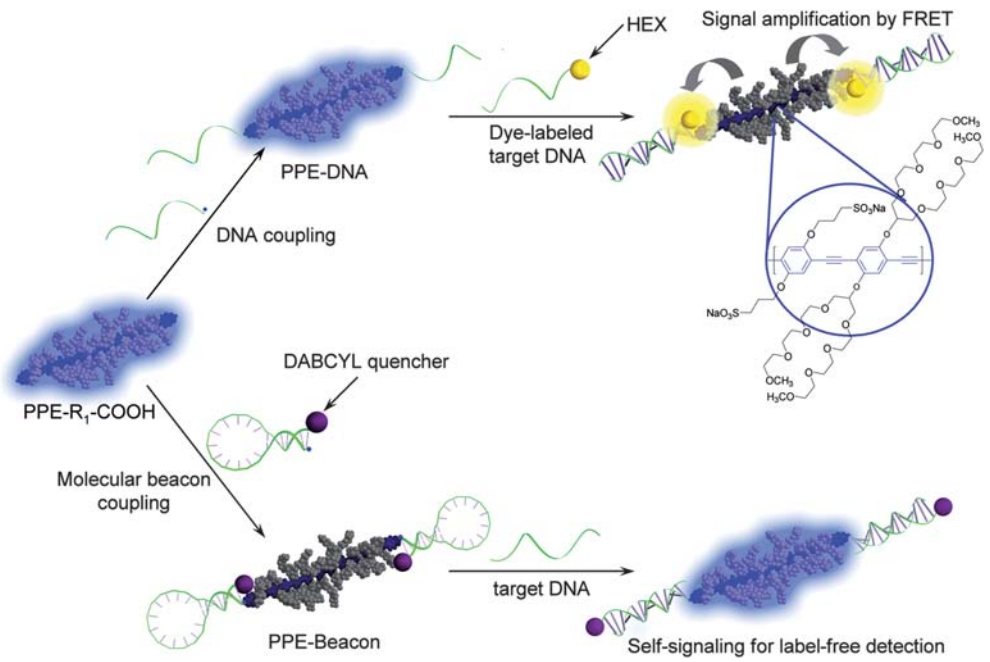

Fig. 2 Pathways for DNA detection using bio-conjugated CP. Top: signal amplification and fluorescence color change *via* FRET from polymer to dye-labeled target DNA. Bottom: polymer fluorescence revival after target DNA coupling with quencher-labeled molecular beacon (reproduced with permission from ref. 43).

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cationic polymer due to their electrostatic interactions. Wang *et al.* strived to improve the specificity of the polymer/DNA sensor by using a common intercalating dye, ethidium bromide (EB).[69] The same system as the previous polymer/DNA/DNA-dye triplex method was used except EB was introduced and the sensing mode was based on the emission of EB. When target complement undergoes specific hybridization EB is selectively intercalated into the hydrophobic regions of the double helix. Large signal amplification of EB is provided by a stepwise energy-transfer process, which the authors call a fluorescence resonance gate (FRET gate), from polymer to fluorescein (FRET1) and then from fluorescein to EB (FRET2). The cascading energy-transfer process provides a substantial increase in EB emission through the light harvesting and signal amplifying properties of the CP. FRET studies have also been reported between CPEs and the intercalating dye PicoGreen, which is has a larger absorption coefficient than EB. CPE DNA sensors with PicoGreen have a detection sensitivity that is improved by a factor of almost 20.[70] In addition, Pu and Liu covalently bonded the intercalating dye thiazole orange (TO) to a cationic poly(fluorene-*alt*-phenylene) CP to differentiate double-stranded DNA (dsDNA) from ssDNA in serum.[71] When only ssDNA is present TO is free to rotate and is only weakly fluorescent. Conversely, when dsDNA is in solution TO is fixed in place and is able to fluoresce. Since TO is covalently bonded to the CP, FRET can occur from the CP to TO when the solution is excited at 370 nm. This results in a large signal amplification and fluorescence color change upon increasing concentration of dsDNA.

Detection methods that do not require modification or labeling of the target molecule are desirable for their convenience. Tan and co-workers reported a *turn-on type* DNA-PPE conjugate for label-free DNA detection.[57] An oligonucleotide unit that can form a molecular beacon hairpin loop was first synthesized on fluorescent quencher (DABYCYL = 4-(4-(dimethylamino)phenyl-azo)benzoic acid)-terminated controlled pore glass (CPG). The oligonucleotide on the CPG support was chemically modified with an iodine group, making it active as a monomer for the polymerization of PPE. *In situ* polymerization using two monomers, diethynylbenzene and diiodo-benzene with a sulfonated ionic pendent group, was achieved in the presence of the iodine-modified oligonucleotide CPG support. After the polymerization unbound PPE was easily separated from the DNA-PPE product by washing and centrifugation and the DNA-PPE hybrid materials were cleaved and deprotected to produce the final product. When the molecular beacon is in its closed state the polymer chain is close to the quencher and the emission from PPE is strongly suppressed. In the presence of complementary DNA the beacon opens and the PPE is able to emit an amplified fluorescence signal. Since PPEs aggregate in water and their emission is red-shifted due to the hydrophobic nature of the polymer backbone, non-ionic surfactant was needed to induce complete water-solubility. The level of water solubility of CPEs critically influences the sensitivity of the solution-state sensors. Kim *et al.* overcame the solubility problem by using bulky, hydrophilic ethylene oxide side chains on the PPE.[43,72] Liu and coworkers have also developed two cationic polyfluorenes (PFs) with ethylene oxide side chains for fluorescence turn-on heparin sensors that have different water solubilities. PFs that are more soluble in water show a lower fluorescence background and better sensitivity.[73]

CPE biosensors have also been developed to detect small biological molecules. Wang and coworkers reported the use of guanine(G)-rich dye-labeled DNA molecules for potassium detection. Prior to adding potassium, the cationic CP-DNA complex has weak emission. When potassium is added, each G-rich DNA forms a quadruplex around an ion and induces a conformational change in the polymer. This results in efficient FRET from the polymer to the dye.[74] In a similar manner, anionic conjugated polyfluorene quenched by metal (Fe) ion-containing hemin showed fluorescence enhancement in the presence of aptamer nucleic acid, which can stably bind with hemin.[75] The hemin-aptamer complex induces separation of CPEs from Fe ions, resulting in the fluorescence revival. He *et al.* have also used CPEs to sense glucose through the detection of H_2O_2.[76] The CPE used is covalently linked to peroxyfluor-1, a non-fluorescent, protected form of fluorescein. The authors take advantage of the production of H_2O_2 when glucose is oxidized by glucose oxidase, which results in cleavage of the protecting group on fluorescein. Therefore, when all three components are in solution, the fluorescein is able to fluoresce. Since fluorescein is covalently liked to the CPE, FRET can occur from the CPE to fluorescein, thus amplifying the signal and making detection in serum possible.

The G-quadruplex-to-duplex transition has also been used to study the cascade energy transfer between a fluorescein-labeled guanine(G)-rich oligonucleotide probe, EB and CP.[77] Before hybridization G-rich DNA forms a stable quadruplex with the aid of potassium ions and is attached to a cationic polymer through electrostatic interactions. Even though FRET1 from polymer to fluorescein happens, fluorescein and EB are not close enough for FRET2 to occur. However, the quadruplex-to-duplex transition in the presence of target complementary DNA enables EB to intercalate the double helix. Close proximity of the polymer/fluorescein-DNA to EB provides an effective FRET gate for the detection of amplified EB emission. The overall process provides substantial improvement in terms of specificity over previous homogeneous CP-based DNA sensors that use FRET sensitization.

CPE-based fluorogenic probes for proteases have been developed by Swager and co-workers.[78] They synthesized a set of carboxylic acid-functionalized PPEs in which the carboxylic groups are separated from the polymer backbone by ethylene oxide spacers to make the polymer water-soluble. An oligopeptide that has a sequence specific to the protease trypsin is covalently conjugated to the carboxylic acids of PPE through the use of carbodiimide chemistry. Also, dinitroaniline quenchers added to the end of the peptide unit can effectively quench the fluorescence of the PPE. After the peptide is cleaved by trypsin the PPE fluorescence is restored. Although the polymer is soluble in water and organic solvents, it is still prone to aggregation in water, which induces fluorescence quenching and a low quantum yield. However, the rate of fluorescence turn-on is increased 3-fold by using a surfactant while the actual rate of enzymatic cleavage remains the same. The cleavage of DNA by nucleases can also be monitored using FRET by observing fluorescence changes in CPs or fluorescein. Wang *et al.* reported the use of a CP-DNA-fluorescein complex to assay nucleases and

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methyltransferases.[79] They furthermore applied this concept to acetylcholinesterase-acetylcholine protease detection.[80] The sensitivity of detection was enhanced by FRET from the CP when compared to the conventional UV/Vis spectroscopy method. In addition, sulfonated polyphenylenevinylene has been used to observe FRET quenching from polymer to ruthenium (Ru)-tethered biotin.[81] The efficiency of FRET between Ru and polymer was controlled by the avidin-biotin interaction.

Several approaches using CPEs for live cell imaging have been demonstrated. Live cell imaging using negatively charged carboxylated PPE was conducted by Fahrni and coworkers.[82] They observed prolonged internalization of anionic CPEs into fibroblast cells and selective filamentous staining of fibronectin. Conversely, cationic PPEs with quaternary ammonium functional groups showed punctate staining. Pu *et al.* also developed core-shell nanoparticles with a hyperbranched CPE core and a cytocompatible poly(ethylene glycol) shell. The particles can be internalized by cells and demonstrate photostability and strong fluorescence within the cell cytoplasm.[83] Cell-staining nanoparticles have also been developed that contain silsesquioxane cores and CPE shells.[84] Besides having inherent blue fluorescence, the nanoparticles can amplify the red emission of EB *via* FRET when dsDNA is present. Therefore, when the nanoparticles and EB are internalized by cells they emit blue fluorescence in the cytoplasm and red fluorescence in the nuclei through FRET. Bunz and coworkers reported the use of cationic gold-nanoparticles with anionic CPEs to detect bacteria.[85] The polymer-nanoparticle composites serve as the recognition element and signal transducer. After the addition of bacteria, the fluorescence from CPEs, which is originally quenched by FRET from polymer to gold nanoparticles, is recovered. Through charge-charge interactions, positively charged, hydrophobic gold nanoparticles have a stronger attraction to negatively charged bacteria than to the CPEs. Therefore, when bacteria are present, the CPEs are detached from the gold nanoparticles and their fluorescence is recovered. The degree of fluorescence recovery varies based on the type of bacteria being tested. Furthermore, they have recently investigated the correlation between the fluorescence quenching efficiency and surface functionality of gold nanoparticles.[86] Rotello *et al.* have developed another system that differentiates between the normal and cancerous cell lines.[87] Nanoparticle-polymer conjugate probes show a selective response to subtle changes in the physicochemical nature of different cell surfaces. The different degree of interaction between the cells and probes induced transduction signals with different fluorescence intensities.

Another strategy that has been successfully implemented is the modification of CPEs with side chains that contain functional groups associated with live cells. Li *et al.* have functionalized CP particles with folic acid in order to increase their uptake by cancer cells compared to non-cancerous cells, thus increasing their level of fluorescent staining.[88] Also, the detection of *Escherichia coli* (*E. coli*) has been reported through the use of carbohydrate-functionalized PPE.[89] Side-chain modification of water-soluble carboxylated PPE with 2′-aminoethyl mannoside units was implemented because cell surface carbohydrates are used as receptors by many kinds of pathogens that cause human infections. When mannose-functionalized PPE is incubated in Alexa Fluor 594(yellow dye)-labeled concanavalin A (Con A), a lectin that binds mannose, the strong blue fluorescence from PPE is quenched through FRET from the polymer to the dye. A control galactose-functionlized PPE does not show any fluorescence quenching, which confirms that mannose-functionalized PPE has specificity toward the lectin. Also, incubation of a mannose-functionalized PPE with *E. coli* yields brightly fluorescent aggregates of bacteria. This aggregation is due to multivalent interactions between the carbohydrate receptors on the *E. coli* pili and the glycosylated PPE. Design of polymer-based detectors for other pathogens only requires information on the carbohydrates that they bind, which has been extensively reported in the literature. Another similar report was recently published that synthesized mannose-bearing CPEs *via* post-functionalization. The authors also demonstrated efficient detection of E. Coli and the potential application for bacterial imaging.[90]

Nilsson and co-workers have also shown that CPEs can be used to stain protein aggregates in tissue that are associated with disease states.[91,92] Derivatives of charged polythiophenes have been developed that bind non-covalently to amyloid fibrils, pathological markers for Alzheimer's disease. The fibrils can take on varying conformations and the CPEs appear to take on these conformations when they are bound. Since the CPE fluorescence emission can be altered based on its conformation, the different amyloid structures are stained different colors by the CPEs. This development has interesting implications for the study of the pathology of Alzheimer's and other protein aggregate diseases.

Solid-state sensors

Fluorescence based solid-state DNA or protein detection using polymer-grafted microparticles or microarrays is a growing trend in biosensor technologies. Solid-state DNA/protein sensors have many advantages such as fast and facile handling and low-cost when compared with homogeneous solution-based sensors that require fastidious isolation steps. In 2004 Whitten and co-workers reported a highly sensitive protease sensor using fluorescent CPE superquenching techniques.[58] They prepared a synthetic peptide substrate that contains recognition and cleavage sequences along with a non-fluorescent quencher (QSY-7 or Azo) and biotin at the opposite end. Also, microsphere sensors were prepared by coating polystyrene microspheres with biotin binding protein (streptavidin or neutravidin) followed by anionic biotin-functionalized PPEs or cationic PPEs. Quencher and biotin-tethered oligopeptides were incubated in an enzyme solution and the fluorescent polymer-coated microspheres were added after the enzymatic reaction. This approach provided a very sensitive assay for cleavage in the presence of enzyme. Microspheres showed intense emission when enzymatic oligopeptide hydrolysis cleaved the tether between biotin and the quencher. In another approach, oligopeptides were first mixed with polymer-coated microspheres and then the enzyme was added. However, this approach resulted in fluorescence quenching of the polymer because enzymatic cleavage was inhibited when the peptide was anchored on the microspheres.

Fan and coworkers have demonstrated the use of magnetic particles that significantly improve the selectivity of CP-DNA conjugates.[93] Capturing oligonucleotides with a specific sequence were tethered to magnetic microparticles and were mixed with target DNA and signaling probe DNA molecules that were labeled with fluorescein. The target DNA molecules have a sequence that matches the capturing DNA on one end and

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a sequence that matches the signaling DNA on the other end. Annealing the solution induces the hybridization of conjugate particle-DNA with target and signaling probe DNA, resulting in the formation of sandwich complexes. Further isolation of target DNA was then achieved by applying a magnetic force to remove the unbound mismatch DNA. Therefore, the magnetically assisted assay can conveniently eliminate nonspecific interactions of mismatches in the target sequence.

In an attempt to develop practical DNA detection systems, a CP-based signal amplifying solid-state DNA sensor[94] and highly sensitive DNA hybrid chips[46] that use on-chip DNA synthesis and the signal amplifying ability of CPs were reported. There are several advantages to on-chip oligonucleotide synthesis, such as its parallel fashion, flexibility in sequence design, and easy manufacturing process. Also, it has high sequence fidelity when compared with other recently developed methods, such as pin micro-dotting, ink-jet microdropping and electrostatic addressing. On-chip DNA synthesis technologies, however, require rigorous conditions such as UV irradiation and strong acid to make the microarray patterns directly. Several different types of CPs, PPEs, poly(thiophenes), and poly(oxadiazoles), were designed and synthesized to test their stability against these harsh conditions.[95] Oxadiazole–containing CPs remain intact throughout the harsh treatments and have strong photoluminescence with an emission maximum at 420 nm. The two other polymers have severely damaged conjugated backbones and lose their emissivity. Oxadiazole is an electron-poor heterocyclic molecule that has been used for polymer designs in which polymer stability is required. Also, the polymer contains an amine group, which can be used for the immobilization of the polymer to a glass substrate and for on-chip DNA synthesis. The on-chip DNA synthesis on the polymer-coated glass substrate was conducted using a modified automatic oligosynthesizer equipped with a UV patterning device. The density of oligonucleotides on the polymer-coated surface is similar to the density on a conventional control slide. In an assay test, hexachlorofluorescein (HEX) labeled complementary DNA was used to observe FRET from the polymer to the HEX dye. The polymer has good spectral overlap with HEX, satisfying the requirement for effective FRET. Upon hybridization and excitation of the polymer at 380 nm, large signal amplification from HEX emission is observed and its intensity is 10-fold greater than the intensity from direct excitation of HEX at 535 nm. The signal-amplifying DNA chip device noticeably lowered the detection limit for target DNA to sub-picomolar concentrations. The technique demonstrates a fast and readily applicable strategy for making signal-amplifying DNA microarrays by combining a CP with efficient and convenient on-chip DNA synthesis.

The above technique has been further developed for label-free detection of DNA. Efficient FRET has been demonstrated from a surface tethered CP-DNA complex to the intercalating dye SYBR green upon addition of complementary DNA. Like previous systems, FRET provides large signal amplification of the dye.[45] Another approach for label-free detection of DNA was recently achieved using molecular beacons. Several different sequences of oligonucleotides that form a hairpin-loop structure and are end-functionalized with quenchers or dyes have been directly synthesized on CPs that are tethered on a substrate. After DNA hybridization with a label-free target complement, fluorescence enhancement or quenching was observed.[44] Label-free DNA detection can also be achieved through the use of PNA microarrays, similar to PNA solution-state sensors.[96] PNA strands are immobilized on an amine-modified glass surface and treated with ss-DNA. If the ss-DNA is complementary, it will bind with the PNA, creating a negatively charged surface. When cationic CPE is added to the system it binds electrostatically to the DNA/PNA complex, thus highlighting the target DNA. To enhance the signaling, a reporter fluorophore can also be attached to the PNA strand. After excitation of the CPE, FRET can occur from the CPE to the fluorophore.[97] The results of the label-free systems provide design principles for the future development of self-signal-amplifying DNA microarrays that could provide PCR-free DNA detection.

Although most label-free solid-state sensors have focused on DNA detection, label-free protein sensors have also been developed through the use of DNA aptamers and cationic polythiophene. Abérem *et al.* prepared a solid-state, fluorescent protein sensor through the use of a ssDNA aptamer specific to the protein thrombin.[98] The aptamer was bound to a glass surface and labeled with a dye that has spectral overlap with the cationic polythiophene used in the assay. Initially, the polythiophene forms a non-fluorescent duplex with the aptamer. When thrombin is added the fluorescence of the polythiophene is revived and the CP transfers this energy to the dye *via* FRET. The use of other aptamers in this type of system could result in the detection of many different types of proteins.

Colorimetric conjugated polymer biosensors

As discussed in the previous section, fluorescent CPs are able to detect extremely small quantities of analyte due to their ability to amplify sensory signals. Although fluorescence sensing is ideal for detecting extremely dilute concentrations of biological molecules, it requires the use of expensive spectrophotometer instruments. The most convenient method for detecting biological molecules is colorimetric sensing since the signal can be seen without using equipment. The two major types of colorimetric CP sensors discussed in this section are those based on polythiophenes and polydiacetylenes (PDAs). These polymers change color when their conformation is altered and various derivatives have been synthesized to sense small biological molecules along with protein, DNA and cells.

Solution-state sensors

Many solution-state colorimetric biosensors have been made using polythiophene derivatives. Leclerc and co-workers first synthesized water-soluble biotinylated poly(3-alkoxy-4-methylthiophene)s to demonstrate their sensing ability.[99] The polymer has a violet color in solution but turns yellow when avidin is added. When biotin binds to the large avidin protein the polymer main chain is twisted, which shortens the conjugation length of the backbone and changes the color of the solution. Ho *et al.* also used poly(3-alkoxy-4-methylthiophene)s for label-free colorimetric detection of DNA.[34] In this system a water-soluble cationic polythiophene is first exposed to single-stranded DNA. Since the ssDNA is negatively charged, it forms a duplex with the polymer

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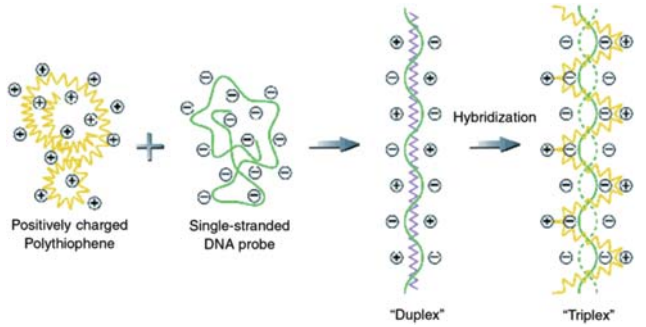

Fig. 3 Label-free colorimetric sensing of oligonucleotides using a water-soluble polythiophene (reproduced with permission from ref. 34).

and causes the polymer chain to maintain an extended conformation (Fig. 3). This change in conformation causes the originally yellow polymer solution to turn to red. After the complementary DNA strand is added, the solution turns back to yellow because of the formation of a triplex conformation. In this state, the polymer is less planar than in the duplex conformation, and thus has a shorter conjugation length and lower wavelength absorption. Using simple electrostatic interactions and subsequent conformational changes the authors were able to colorimetrically sense oligonucleotides without any chemical labeling at a detection limit of 2×10^{-7} M. It is worth noting that the cationic polythiophene used in this experiment is also fluorescent and the fluorescence is quenched in the duplex (planar and aggregated) state. The fluorescence is mostly recovered in the triplex conformation and this can be used to decrease the detection limit as low as 2×10^{-14} M.

After Ho *et al.* demonstrated the detection of ssDNA with cationic polythiophene, other nucleotide sensing systems have been developed. For example, Tang *et al.* used a cationic polythiophene to detect the enzymatic and hydroxyl radical cleavage of ssDNA.[100] The ssDNA initially forms a duplex with cationic poly[3-(3′-N,N,N-triethylamino-1′-propyloxy)-4-methyl-2,5-thiophene hydrochloride] (PMNT). After cleavage of the DNA the duplex is disrupted and the cationic polythiophene adopts a random coil conformation. This change in conformation results in a blue shift of solution absorption and a color change from pink-red to yellow. This type of assay has the potential to examine the efficiency of cleavage enzymes and also to test drugs that are developed to prevent the degradation of DNA. More recently, Zhang *et al.* took this concept further by using cationic polythiophenes to detect microRNA and the enzyme activity of RNase H.[101] The system uses PMNT to first bind with ssDNA, which results in the extended conformation for PMNT. When complementary microRNA is added, the PMNT forms a triplex with the ssDNA-microRNA and the color of the solution changes from yellow to orange. If RNase H is added to digest the microRNA, the PMNT reforms a duplex with the ssDNA and the solution absorption becomes red-shifted. Another recent development has been the detection of 15 different nucleotide phosphates using a cationic poly(3-alkoxy-4-methylthiophene) derivative.[102] Since each nucleotide phosphate has either a different number of phosphate groups (monophosphate, diphosphate or triphosphate) or varying degrees or hydrophobicity, they each have different electrostatic and secondary bonding interactions with the cationic polythiophene. Therefore, the polythiophene conformation and aggregated structures are different for each nucleotide phosphate and this is reflected in the absorption spectra of the polythiophene solution. The resulting solutions are varying shades of purple, pink, yellow and orange. Although the discrimination between all of the nucleotide phosphates is novel, the paper is based off of previous work by Li *et al.* to detect ATP with the same cationic polythiophene.[103]

Solution biosensors that detect proteins and small biological molecules have also been developed using water-soluble polythiophenes. Ho and Leclerc developed a system to detect human α-thrombin using an aptamer that specifically binds to the protein.[104] Since the aptamer also binds to cationic polythiophene through charge-charge interactions, the addition of α-thrombin results in a polymer conformational change and, subsequently, color change. Colorimetric sensors have also been developed to detect inorganic phosphate anions, which are important in many biologic processes. Pyrophosphate and phosphate anion sensors have been developed based on polythiophenes that are copolymerized with dipyrrolylquinoxaline (DPQ) units.[105] DPQ can form hydrogen bonds with small anions, and as a result, the polymer conformation changes when phosphate anions are present. Like the previous systems, a color change occurs when phosphate anions are present and the authors also measured a change in conductivity due to the doping effect of the anions on the CP backbone.

The CP polydiacetylene has also been used to develop solution-state colorimetric biosensors, although more recent developments have focused on making convenient solid-state devices, as discussed in the next section. Diacetylene (DA) monomers are typically synthesized as a diacetylene moiety with hydrophilic and hydrophobic tails, which allow the molecules to self assemble into liposomes or bilayers. After self-assembly, the monomers are polymerized using UV irradiation at 254 nm, thus forming liposome polymer structures that have a blue color. Interestingly, when subject to external stimuli such as temperature change,[106,107] pH change,[108,109] or mechanical stress,[110,111,112] the PDA liposomes change from blue to red due to a conformational change of the conjugated ene-yne backbone of PDA. The main quantitative measurement used to describe PDA sensing is the colorimetric response:

$$\mathrm{CR} = (\mathrm{PB}_0 - \mathrm{PB}_f/\mathrm{PB}_0) \times 100\%$$

where PB_0 and PB_f are percent blue before and after the color transition as defined by

$$\mathrm{PB} = \mathrm{A}_{640\ \mathrm{nm}}/[\mathrm{A}_{640\ \mathrm{nm}} + \mathrm{A}_{550\ \mathrm{nm}}]$$

where A is absorption. More in-depth reviews of PDA chemical and biological sensors have been published recently that describe a broader range of PDA chemical and biological sensors.[113–116]

Unlike cationic polythiophene sensors, most PDA biosensors involve the incorporation of specific receptors into the polymer or liposome. When a target molecule binds with the receptor, steric forces can change the PDA backbone conformation, resulting in the color change from blue to red. For example, Wang and Ma attached complementary oligonucleotides to two separate suspensions of PDA liposomes and showed that mixtures of these liposomes change from blue to red.[117] Since

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single stranded oligonucleotides are relatively short and linear, the binding event of unmodified complementary DNA may not create enough perturbation in the liposome to cause a color change. The attachment of PDA liposomes to both strands of DNA creates much more steric repulsion upon DNA hybridization, which results in enough conformational change that the suspensions change color. The detection limit of the system is around 20 nM. Jung *et al.* took a different approach in developing universal sensors for nucleic acids.[118] Rather than attaching specific oligonucleotides to the liposomes, the system uses the ionic interactions between amine-functionalized PDA and negatively charged DNA to perturb the PDA backbone. Although this method cannot detect specific sequences of DNA, it does allow for very easy detection of any nucleic acid strands, which could be useful for determining the presence of DNA after PCR.

Receptor-based PDA solution sensors have also been used to detect proteins. Recently, calixarenes that bind to specific amino acids were embedded in lipid/PDA vesicles in order to colorimetrically distinguish different proteins from each other.[119] The initial proteins tested – pepsin, histone, and albumin – each had different colorimetric effects on the liposomes since the proteins have different electrostatic interactions with the calixarenes and the other liposome components. By investigating a larger set of proteins, it was shown that the colorimetric responses of the liposomes were related to the isoelectric points of the proteins tested. The authors also plotted the colorimetric responses of liposomes with two different calixarenes against each other for the larger set of proteins. Each protein had a different marking on the plot, thus indicating that this method could be used to identify a colorimetric fingerprint for individual proteins. In addition to proteins, solution PDA sensors with covalently bonded or embedded receptors have also been used to detect antibody-epitope binding,[120] phosphate ions,[121] α-cyclodextrin,[122] lipopolysaccharides[123] and bacterial cells.[124]

Solid-state sensors

The first PDA biosensor, developed by Charych *et al.*, was a solid-state PDA bilayer used to detect the influenza virus.[125] Since this initial design, many convenient solid-state platforms have been developed to colorimetrically detect biomolecules. One method for immobilizing PDA liposomes is encapsulating them with another material.[126–129] For example, PDA liposomes can be embedded in agarose gel to detect the presence of bacteria by interacting with molecules secreted by the bacteria.[127,129] As a result, the matrix color is blue and red patches appear where bacteria colonies are present. Also, *E.coli* bacteria can be detected using PDA sol–gel nanocomposites.[126] The PDA liposomes were functionalized with various antibodies, including anti-*E.coli* β-galactosidase, and encapsulated in sol-gels consisting of silica, siloxanes and poly(vinyl alcohol). Although encapsulation does decrease the response time of the sensor and the total CR, the functionalized PDA could detect 10 ng/ml solutions of E.coli β-galactosidase with a CR near 75% within 10 min. In addition to β-galactosidase, the liposomes were functionalized with antibodies that could detect α-fetoprotein, bovine serum albumin and yeast alkaline phosphatase.

Besides encapsulation, PDA liposomes can also be covalently immobilized on substrates by incorporating DA monomers that have reactive functional groups.[130] Kim *et al.* attached PDA liposomes to aldehyde-functionalized glass through the use of amine-terminated DA monomers in the creation of PDA liposomes. The best results were obtained when the unpolymerized liposomes were first reacted with the functionalized glass to form an imine link and then were polymerized with UV irradiation. This method produces a monolayer of liposomes that exhibit a blue color and thus can be reacted with a stimulus to function as a sensor. Other groups have used similar methods of covalent attachment to form thin film or microarray PDA sensors to detect cyclodextrins,[131] bacteria,[132,133] potassium ions[134] and mercury ions.[135]

While the colorimetric signal of PDA is convenient, it does not allow scientists to detect extremely small quantities of analyte. This problem can be overcome, however, since the red form of PDA is fluorescent and the blue phase is not emissive.[106] Platforms that can utilize the fluorescent properties of PDA have been studied for solid-state[131,136] and solution-state sensors,[137] although most recent work has used immobilized liposomes. Lee *et al.* developed PDA liposomes that are covalently bound to G-rich ssDNA aptamers, which specifically fold around potassium ions and form quadruplexes.[134] The resulting bulky quadruplexes repulse each other and impose stress on the conjugated backbone of the PDA liposome. The liposomes change to the red state and are fluorescent when the ssDNA binds to potassium (Fig. 4). Microarrays of these liposomes can detect concentrations of potassium that are lower than physiological levels, even in the presence of large concentrations of sodium. Also, fluorescent microscope images can be used to quantitatively measure the concentration of potassium between 0.5 mM–50 mM. In another study, Park *et al.* amplified the fluorescence of PDA by crosslinking liposomes with diethylamine to form multilayer films.[133] Crosslinking largely increases the number of liposomes on the surface and increases the fluorescence of the films by more than 10 times when compared to films made without diethylamine linker. By attaching antibodies to the PDA liposomes, the authors were able to detect six different pathogens and quantitatively measure concentrations from 10^2–10^6 cells/ml. In addition, multiple pathogens were sensed on a single sensor by isolating liposomes with different antibodies in specific areas on the sensor.

Future outlook

As the recent progress is summarized in this review article, the fluorescent and colorimetric CP-based biosensors have attractive merits as a sensory system and have expanded their applications to various biomolecular detections. An excellent sensory system must provide high sensitivity and high selectivity together with the capability for convenient label-free detection. The signal amplification principle by means of the macromolecular response of a CP chain has been well established to achieve high sensitivity. This 1-dimensional amplification scheme has been further expanded to 2-dimensional and even 3-dimenstional amplification schemes through directed molecular assembly of CPs into nanofibers, well-defined films, and three dimensional objects.[138,139] When it comes to high selectivity, devising rationally designed receptors that have specificity toward a target analyte is essential. Convenient and effective strategies such as the use of aptamers

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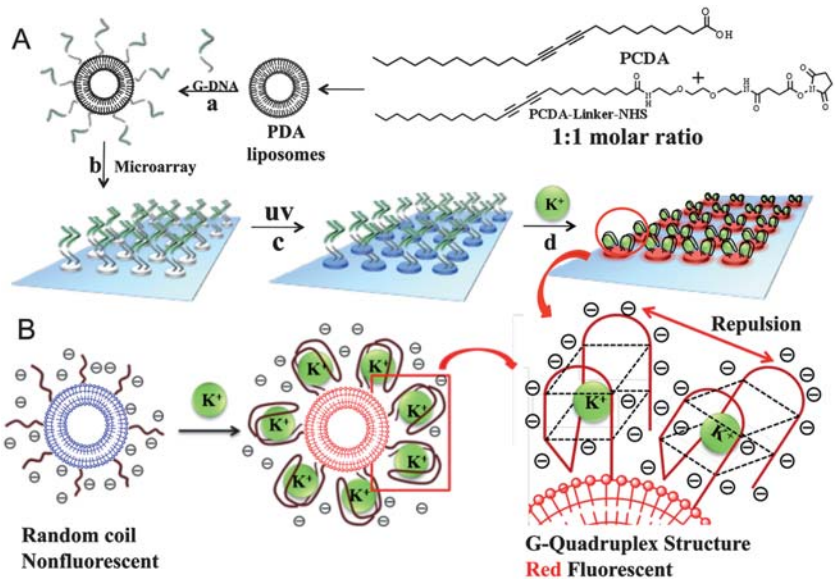

Fig. 4 Scheme for sensing potassium ions using PDA liposomes labeled with guanine-rich ssDNA. The liposomes are immobilized as microarrays and the spots change from blue to red when potassium is present (A). When the ssDNA folds around the potassium ions there is steric repulsion between DNA strands, which disrupts the PDA backbone and results in red color and fluorescence (B) (reproduced with permission from ref. 134).

and molecular imprinting of synthetic receptors are promising directions in this regard. Clever molecular designs that provide label-free sensing are necessary for rapid identification and detection of target molecules without pre-treatment of analyte samples. As summarized in this review various strategies have been developed or are under investigation that utilize conformational change or molecular aggregation of CPs induced by analyte recognition.

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