Optical Emission of a Conjugated Polyelectrolyte: Calcium-Induced Conformational Changes in Calmodulin and Calmodulin−Calcineurin Interactions

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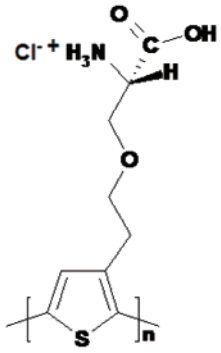
ABSTRACT: Electronic polymers in aqueous media offer bioelectronic detection of biomolecular processes. Here we report fluorometric detection of calcium-induced conformational changes in calmodulin based on noncovalent assembly of calmodulin to a water-soluble zwitterionic polythiophene derivative. Assembly with calmodulin will induce a planar geometry and aggregation of the polymer chains, detected as a decrease of the intensity and a red shift of the fluorescence. Upon addition of Ca^{2+} the intensity of the emitted light is increased and blue-shifted. The geometrical alteration of the polymer chains can further be utilized for recording of the binding of calcineurin to the calcium-activated POWT−calmodulin complex. This novel methodology, using a conformation-sensitive probe, allows fluorometric detection of conformational changes in biomolecules and protein−protein interactions without any covalent modifications of the biomolecules. The rapid and selective method is based on noncovalent interactions between a zwitterionic polythiophene derivative and the biomolecule of interest. This offers a novel way to create microarrays without using covalent attachment of the receptor or labeling of the analyte.

Introduction

Materials showing recognition properties toward biological molecules, such as ions, proteins, and nucleic acids, have come under increasing attention, owing to their large potential for biomolecular electronics and biosensors. Conjugated polyelectrolytes (CPs) such as polythiophene and polypyrrole are capable of continuously and selectively detecting biomolecular interactions and have potential for being used as biosensors and in molecular electronics.[1−8] A wide range of CPs have been used as detecting elements for biological molecules in an aqueous environment, and many of these systems utilize the impact of biomolecules on the conditions for photoinduced charge or excitation transfer.[9−11]

Previous studies[12−14] of poly(3-[(*S*)-5-amino-5-carboxyl-3-oxapentyl]-2,5-thiophenylene hydrochloride), POWT (Figure 1), have shown optical phenomena due to geometrical changes within the polyelectrolyte chain and between adjacent polymer chains upon exposure to biomolecules. The interactions between the polymer and biomolecules are causing different conformational transitions of the polyelectrolyte backbone and aggregation or separation of the polyelectrolyte chains.[12−14] The functional groups of the zwitterionic side chain, anionic or cationic at different pH, make this polythiophene derivative suitable for forming polyelectrolyte complexes with negatively or positively charged oligo- and polyelectrolytes. In addition, the zwitterionic groups are able to create versatile hydrogen-bonding patterns with different molecules, thus offering a new route for conjugated polymer/biopolymer interactions.

Conformational changes of biomolecules are very important in biological systems, forming part of the chain of molecular interactions leading to signal transduction in the cell. Calmodulin (CaM) is a small protein (148 amino acids) that functions as the primary intracellular calcium sensor in eukarotic cells and plays an essential role in calcium-mediated signal transduction.[15]



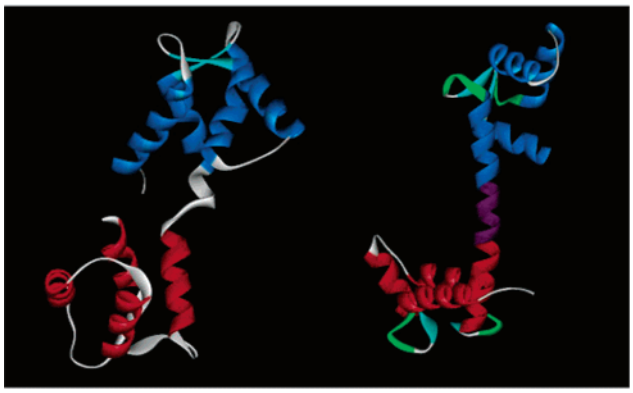


Figure 1. Repeating unit of poly(3-[(*S*)-5-amino-5-carboxyl-3-oxapentyl]-2,5-thiophenylene hydrochloride) (POWT) (top). Ribbon diagram of apo CaM (coordinates: 1CFD.pdb)[18] (bottom left) and ribbon diagram of $(Ca^{2+})_4$ −*Parameicum* CaM (coordinates: 1osa.pdb)[22] (bottom right).

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Table 1. Absorption Maximum, Emission Maximum, and the Ratio of the Intensity of the Emitted Light at 540/670 nm for Solutions Containing Different Amounts of POWT, Calmodulin, and Ca^{2+}

	abs max (nm)	emission max (nm)	ratio of the intensity of the emitted light at 540/670 nm
POWT	421	565	3.87
POWT + 10 mM Ca^{2+}	421	565	3.84
POWT + 1.2 nmol of calmodulin	455	608	0.62
POWT + 1.2 nmol of calmodulin + 10 mM Ca^{2+}	440	601	1.91

The overall structure of CaM (Figure 1) consists of two globular calcium-binding domains, each containing two calcium-binding regions with the characteristic EF hands,[16] connected by a linker. Upon binding of calcium, the relative orientation of the two α-helices that define the EF hand changes substantially, resulting in a transition from a closed to an open conformation of the motif. Structural studies have also shown that calcium activation of CaM is accompanied by a global conformational change, whereby the compact calcium-free form of CaM is converted to a more extended dumbbell-shaped molecule upon binding of calcium.[17,18] The extended form of the protein consists of two lobes separated by a central α-helix, and this central helix is flexible and allows considerable movements of the two lobes with respect to one another.[19−21]

The conformational flexibility of conjugated polyelectrolytes allows direct correlation between the geometry of chains and the resulting electronic structure and processes. To use this as a sensor for the recording of conformational changes of biomolecules requires that the conjugated polymer chain geometry will be governed by the conformational changes of the biomolecules. If conformational changes of biomolecules can lead to different conformations of the polymer backbone, an alteration of the absorption and emission properties from the polymer will be observed. Hence, the conjugated polyelectrolyte can be used as a conformation-sensitive optical and electronic probe, appropriate for the making of novel sensors and biomolecular switches. The technique has recently been used to detect the formation of amyloid fibrils of proteins by using the conformational changes of an anionic polythiophene derivative.[23]

In this article we report conformational transitions of a water-soluble, zwitterionic, electroactive, and photoactive polythiophene derivative, induced by noncovalent coupling to CaM, an intracellular signaling protein that alters its conformation upon exposure to calcium. We also observe calcineurin binding to the calcium-activated POWT−CaM complex. A similar study[24] using a cationic polythiophene derivative−single-stranded DNA complex to detect the binding to thrombin was recently reported, so the methodology using noncovalent assembly of a luminescent polyelectrolyte and a biomolecule to detect the presence of a second biomolecule, recognizing the first biomolecule, shows great potential for being used as a new sensor technology.

Materials and Methods

POWT and CaM Binding Studies. The synthesis of the polymer was reported elsewhere.[25] A stock solution containing 4.0 mg of polymer/mL in deionized water was prepared. Lyophilized CaM from bovine brain (Sigma-Aldrich, P2277, mol wt 16 680 Da) was diluted with 20 mM Tris-HCl pH 7.5 to a final concentration of 1.0 mg of CaM/mL. For the absorption and emission measurements 5 μL of the polymer stock solution was mixed with 12.5 μL of 20 mM Tris-HCl pH 7.5 or 12.5 μL of the CaM solution, respectively, and diluted to a final volume of 150 μL with deionized water. After 15 min of incubation, the samples were diluted with a stock buffer solution (Tris-HCl pH 7.5) and 100 μL of deionized water or 100 μL of 0.1 M $CaCl_2$ solution to a final volume of 1000 μL containing 20 mM Tris-HCl. The samples were placed on a rocking table for 10 min before the spectra were recorded. Optical spectra were recorded on a Perkin-Elmer Lambda 9 UV/vis/NIR spectrophotometer for UV/vis and a Hitachi F4500 fluorescence spectrophotometer for fluorescence.

POWT−CaM and Calcineurin Binding Studies. A stock solution containing 4.0 mg of polymer/mL in deionized water was prepared. Lyophilized CaM from bovine brain (Sigma-Aldrich, P2277, mol wt 16 680 Da) and lyophilized human serum albumin (HSA) (Sigma-Aldrich, A1653, mol wt 66 500 Da) were diluted with 20 mM Tris-HCl pH 7.5 to a final concentration of 1.0 mg mL^{-1} CaM and 1.0 mg mL^{-1} HSA, respectively. Lyophilized calcineurin from bovine brain (Sigma-Aldrich, C1907, heterodimer mol wt 77 000 Da) were diluted with 20 mM Tris-HCl pH 7.5 to a final concentration of 1.95 μM. For the emission measurements 5 μL of the polymer stock solution was mixed with 12.5 μL of the CaM solution and diluted to a final volume of 150 μL with deionized water. After 15 min of incubation, the samples were diluted with a stock buffer solution (Tris-HCl pH 7.5), 100 μL of deionized water or 100 μL of 0.1 M $CaCl_2$, different amounts of the calcineurin, and HSA solutions to get the desired concentrations (10, 20, 40, 60, 100, and 150 nM) with a final volume of 1000 μL containing 20 mM Tris-HCl. The samples were placed on a rocking table for 10 min before the spectra were recorded. The emission spectra were recorded on a Hitachi F4500 fluorescence spectrophotometer for fluorescence.

Results and Discussion

POWT and CaM. The absorption maximum for POWT in 20 mM Tris-HCl pH 7.5 buffer solution is shown in Table 1. An absorption maximum of 421 nm, as seen for the POWT in 20 mM Tris-HCl pH 7.5 buffer solution, is associated with a degree of planarization of the polyelectrolyte backbone and is in agreement with the result from earlier studies[12−14] of the polymer in similar buffer solutions. Upon addition of 1.2 nmol of CaM the absorption maximum is red-shifted (Table 1), indicating that the POWT backbone becomes more planar and/or that aggregation of the POWT chains occurs.

The emission spectrum of POWT (93 nmol on a monomer basis) after 10 min of incubation in 20 mM Tris-HCl pH 7.5 is shown in Figure 2. The emission peak at 565 nm is associated with a certain planar conformation of the polyelectrolyte backbone and has previously been seen for POWT in comparable buffer solutions.[12−14] Addition of 10 mM Ca^{2+} will not alter the spectrum (Figure 2), indicating that geometry of the polyelectrolyte chains is not altered up on exposure to calcium.

Upon addition of 1.2 nmol CaM, the emission peak is red-shifted to 608 nm (Figure 2, Table 1) due to planarization of the polyelectrolyte backbone, and the intensity of the emitted light at longer wavelength is also decreased. There is also a shoulder at 540 nm, associated with an intrachain event, which has previously been seen for solutions where POWT adopts a

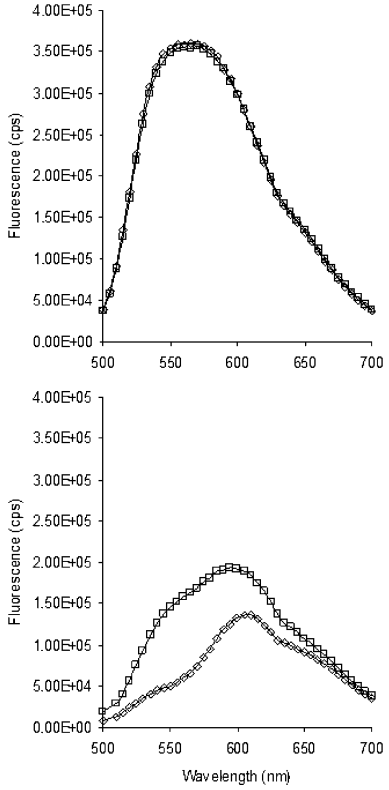

Figure 2. Emission spectra (top) of 93 nmol POWT, on a monomer basis, in 20 mM Tris-HCl pH 7.5 (diamonds) and after addition of 10 mM Ca^{2+} (squares). Emission spectra (bottom) of 93 nmol POWT, on a monomer basis, and 1.2 μM CaM in 20 mM Tris-HCl pH 7.5 (diamonds) and after addition of 10 mM Ca^{2+} (squares).

nonplanar helical conformation[12−14] and a shoulder at longer wavelength associated with an interchain event due to contact between polyelectrolyte chains. These intra- and interchain events have also been observed in thin films of POWT[26,27] and will reduce the fluorescence quantum yield from the single chain level of 26% (observed in dilute methanol solutions) to 4% in solid solutions due to nonradiative deexcitation. This new channel for deexcitation is created in the contact between polyelectrolyte chains, and the emission maximum for the intrachain and the interchain event in thin polyelectrolyte films was 565 and 670 nm, respectively.[26,27] The ratio of the intensity of the emitted light at 540 and 670 nm, 540/670 nm, can be used as a measurement of the geometrical alteration of the polyelectrolyte chains and has previously been used for the detection of biospecific interaction.[13,14] As shown in Table 1, the ratio of the intensity of the emitted light at 540/670 nm is decreased from 3.87 to 0.62 upon addition of 1.2 nmol CaM, indicating that the interaction between CaM and POWT forces the polyelectrolyte chains to aggregate. This agrees with the absorption maximum which is red-shifted, from 421 to 455 nm, upon addition of CaM, indicative for a planarization of the polyelectrolyte backbone and aggregation of polyelectrolyte chains.

We suggest, on the basis of previous studies,[13,14] that the negatively and positively charged groups of CaM will most likely interact electrostatically with the positively and negatively charged groups of the polyelectrolyte side chains. The internal interactions between the amino and carboxyl group are disrupted, leading to a planarization of the polyelectrolyte backbone and aggregation of polyelectrolyte chains. Interestingly, the α-helices of the EF-hand motif (total number of 8) in CaM (Figure 1) contains many negatively charged amino acids, especially aspartate (Asp) and glutamate (Glu), suitable for interaction with the positively charged amino group of the polyelectrolyte side chains. Other nearby amino acids, and the peptide backbone of CaM, are subsequently able to form hydrogen bonding with the polyelectrolyte side chains and thereby stabilize the interaction. On the basis of a previous study of POWT and synthetic peptides[14] and the fact that the POWT molecule most likely adopts a right-handed helical conformation in aqueous solutions,[12−14] the interactions between the polymer and the negatively charged α-helices of CaM are probably the most favorable.

The absorption maximum for the polyelectrolyte in the pure buffer solution (20 mM Tris-HCl pH 7.5) is not influenced by the addition of 10 mM of Ca^{2+} (Table 1). In contrast, an addition of 10 mM mM Ca^{2+} to the solution with the POWT/CaM complex will induce a blue shift of the absorption maxima (Table 1), associated with a planar-to-nonplanar (from highly conjugated to less conjugated) conformational transition of the backbone or a disruption of the aggregated polyelectrolyte chains. Hence, the polyelectrolyte backbone becomes more twisted and nonplanar as CaM changes the conformation upon exposure to Ca^{2+}.

Calcium activation of CaM is accompanied by a global conformational change, whereby the compact calcium-free form of CaM is converted to a more extended dumbbell-shaped molecule upon binding of calcium.[17,18] The relative orientation of the two α-helices that define the EF hand changes substantially, resulting in a transition from a closed to an open conformation of the motif, and a new central α-helix between the two globular domains is also induced upon binding of calcium. All of these events are most likely influencing the geometry of the polyelectrolyte chains, and the recording of these conformational changes is therefore related to the amounts of binding sites of the CaM molecule that are occupied by polyelectrolyte chains. Some MALDI−TOF experiments[28] have shown that the chain length distribution of the POWT polyelectrolyte is between 13 and 19 monomer units. If we use an average chain length of 16 monomer units, the molar relationship between POWT:CaM for the solution will be 8:1. Thus, if the polyelectrolyte chains are interacting with the α-helices of CaM (total of 8), all of the helices will most likely be occupied by polyelectrolyte chains. Hence, all of the different conformational transitions of the CaM molecule are able to govern the geometry of the polyelectrolyte chains and will be reflected as an alteration of the optical properties from POWT. Some experiments, using surface plasmon resonance (SPR) methods, have shown dissociation constants of the same order of magnitude (10^{-7} M) as antibody/antigen interactions (work in progress), suggesting that at micromolar concentrations of CaM and POWT the molecules are mostly existing as the POWT−CaM complex form and not as the free form.

As seen in Figure 2, an addition of 10 mM Ca^{2+} to this solution will blue shift the emission maximum (594 nm) and the shoulder around 540 nm is increasing, suggesting that the polyelectrolyte backbone becomes more nonplanar and that a separation of the polymer chains occur. The ratio of the intensity of the emitted light at 540/670 nm (Table 1) is increased to 1.91,

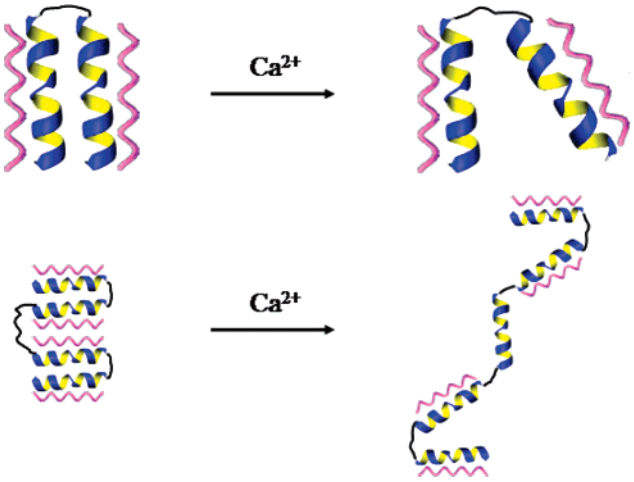

Figure 3. Schematic drawing of the different conformational alterations of the CaM molecule (blue helices) upon exposure to calcium and the suggested POWT chains (pink helices) geometries. The relative orientation of the two α-helices that define the EF hand changes substantially, resulting in a transition from a closed to an open conformation of the motif (top). The global conformational change, whereby the compact calcium-free form of CaM is converted to a more extended dumbbell-shaped molecule upon binding of calcium (bottom).

showing that the conformational change of the CaM molecule upon exposure to Ca^{2+} are governing the geometry of the polyelectrolyte chains. The increased intrachain event at 540 nm, associated with separation of the polyelectrolyte chains, is probably a result of the conformational change whereby the compact calcium-free form of CaM is converted to a more extended dumbbell-shaped molecule upon binding of calcium.[17,18] If the POWT chains are interacting with all of the α-helices of CaM, the relative orientation of the two α-helices that define the EF hand (transition from a closed to an open conformation of the motif) is also most likely influencing the geometry of the polyelectrolyte chains. If there is an excess of POWT molecules in the sample, there is also a possibility that the polyelectrolyte chains are able to interact with the new central α-helix that is created in the calcium binding form of CaM. The geometry alterations of the polyelectrolyte chains are probably a result of all the different events. However, to clarify how the interactions between POWT and CaM occur, some NMR experiments have to be performed.

A schematic presentation of the different conformational alterations of the CaM molecule upon exposure to calcium and the suggested POWT chains geometries seen for the different POWT/CaM solutions is shown in Figure 3. So far we have shown that conformational changes of CaM upon exposure to calcium can be recorded by geometrical changes and alterations of the emission properties of POWT. NMR experiments are in progress to determine the conformational changes in calmodulin that can be recorded by POWT and to determine the interaction between POWT and CaM.

We note that with lower POWT:CaM ratios spectral changes are not as significant or different from those observed at the high POWT:CaM ratio here reported. For instance, with a POWT:CaM ratio 1:1 no spectral changes are observed, indicating that the conformational changes of the CaM upon exposure to calcium are not reflected as an alteration of the geometry of the POWT chains. This result suggesting that there are

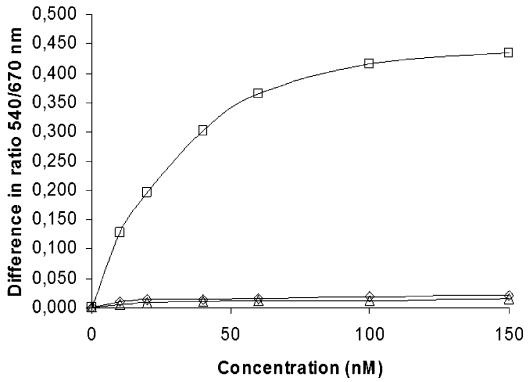

Figure 4. Difference in ratio of the intensity of the emitted light for a POWT−CaM complex upon exposure to different amounts of calcineurin in 20 mM Tris-HCl pH 7.5 10 mM Ca^{2+} (squares), different amounts of calcineurin in 20 mM Tris HCl pH 7.4 (diamonds), and different amount of HSA in 20 mM Tris-HCl pH 7.4 10 mM Ca^{2+} (triangles).

multiple binding site for POWT on the CaM molecule and these sites has to be occupied by the polyelectrolyte molecule to record to conformational alterations of CaM.

POWT−CaM and Calcineurin. Conformational changes of protein and protein−protein interactions are part of the cell-signaling pathways in the cell. We have investigated whether the POWT−CaM complex can be used to evaluate such interactions by exposing the complex to calcineurin, a 77 kDa CaM-binding protein. The titration of POWT:CaM with different amount of calcineurin is shown in Figure 4. The ratio of the emitted light at 540/670 nm is altered with an increasing amount of calcineurin, and the dissociation constant (K_D) for CaM and calcineurin can be estimated to approximately 36 nM. This value is in the same order of magnitude as K_D values obtained with a method using covalently attached fluorescent markers.[29] The ratio of the intensity of the emitted light at 540/670 nm for the POWT−CaM complex is not altered when calcium is not present in the solution. This result is expected, as calcium activation of CaM is needed for the interaction between CaM and calcineurin. Hence, the alteration of the ratio of the intensity of emitted light at 540/670 nm is most likely due to the interaction between calcium-activated CaM and calcineurin. This interaction is probably leading to a separation of the polyelectrolyte chains seen as an enhanced ratio of the intensity of the emitted light at 540/670 nm.

As a control, the calcium-activated POWT−CaM complex was also exposed to human serum albumin (HSA), and interestingly no significant change in the ratio of the emitted light at 540/670 nm could be seen (Figure 4). This result suggests that the unspecific binding between the POWT−CaM complex and HSA is minimal and argues that it is the calcium-activated CaM that is interacting in a selective way with calcineurin. A similar study[24] using a cationic polythiophene derivative−single-stranded DNA complex to detect the binding to thrombin was recently reported. The methodology using noncovalent assembly of a luminescent polyelectrolyte and a biomolecule to detect the presence of a second biomolecule, recognizing the first biomolecule, shows great potential for being used as a new sensor technology.

In conclusion, we have shown that an electroactive zwitterionic polythiophene derivative can be used as an excellent conformation-sensitive probe to record the calcium-induced conformational changes in calmodulin.

The alteration of the optical properties of the polyelectrolyte can further be used to detect the binding of calcineurin to the calcium activated POWT−CaM complex. This novel methodology, using a conformation-sensitive probe, allows fluorometric detection of conformational alterations in biomolecules and protein−protein interactions without covalent modifications of the biomolecules being used. The rapid and selective method is based on different electrostatic interactions between a zwitterionic polythiophene derivative and the biomolecules of interest. The simplicity of this methodology makes it suitable for making inexpensive microarrays for rapid detection of biospecific interactions. We foresee that the present mechanism also may be used for detection of other biospecific interactions and conformational changes of biomolecules and that it may be used to allow electrical detection of these interactions.

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