Direct Molecular Detection of Nucleic Acids by Fluorescence Signal Amplification

ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · OCTOBER 2005

Impact Factor: 12.11 · DOI: 10.1021/ja053417j · Source: PubMed

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Published on Web 00/00/0000

Direct Molecular Detection of Nucleic Acids by Fluorescence Signal Amplification

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Abstract: An integrated PCR-free DNA sensor, which combines a sequence-specific receptor, an optical polymeric transducer, and an intrinsic fluorescence amplification mechanism, is reported. This sensor is based on the different conformations adopted by a cationic polythiophene when electrostatically bound to ss-DNA or ds-DNA, and on the efficient and fast energy transfer between the resulting fluorescent polythiophene/ds-DNA complex and neighboring fluorophores attached to ss-DNA probes. This molecular system allows the detection of only five molecules in 3 mL of an aqueous solution, or 3 zM, in 5 min. Moreover, this work demonstrates, for the first time, the direct detection of single nucleotide polymorphisms (SNPs) from clinical samples in only a few minutes, without the need for nucleic acid amplification.

Introduction

Simple and ultrasensitive sequence-specific DNA biosensors are urgently needed for the rapid diagnosis of infections and genetic diseases as well as for environmental and forensic applications. For instance, the efficient and affordable detection of infectious disease agents is seen by the World Health Organization as the most critical biotechnological development for improving health in developing countries.[1] The recognition capabilities of DNA through hybridization reactions are well-established, but adequate transducers are needed to generate a physically measurable signal from the hybridization event. For this purpose, various optical (molecular beacons, DNA-derivatized nanoparticles, conjugated polymers, etc.) and electrochemical (redox-active nucleic acids, redox polymers, enzymatic systems, etc.) DNA sensors have been proposed.[2−13] Many of them rely on some form of chemical amplification, such as the polymerase chain reaction[14] (PCR), to provide the detection sensitivity and specificity needed for a rapid assessment of the identity of pathogens which might be present in various clinical and environmental samples. However, PCR requires complex mixtures and sophisticated hardware to perform efficiently the enzymatic reaction. To develop particularly simple and inexpensive assays, we report herein a DNA detection system which combines an anionic oligonucleotide probe, a cationic polymeric transducer, which also serves as a localized counterion promoting specific hybridization, and an intrinsic photonic amplification mechanism. For the first time, it is possible to easily and specifically detect as few as five molecules of DNA extracted from clinical samples in 5 min. This approach is suitable for the rapid assessment of the identity of single nucleotide polymorphisms (SNPs), genes, and pathogens without the need for nucleic acid amplification.

Results and Discussion

To develop this direct molecular detection approach, a cationic polythiophene (Polymer 1 in Figure 1) was first synthesized as described elsewhere.[10,13] This water-soluble conjugated polymer exhibits color and fluorescence changes when put in the presence of single-stranded (ss) or double-stranded (ds) nucleic acids. For instance, when a 20-mer unlabeled capture probe (i.e., X1: 5′-CATGATTGAACCATC-CACCA-3′) is stoichiometrically (on a repeat unit basis) added to polymer 1, the fluorescence of the polymer is quenched and the mixture becomes red ($\lambda_{max} = 527$ nm), corresponding to a planar, highly conjugated aggregated form of the polythiophene. Upon addition of a complementary oligonucleotide (i.e., Y1:

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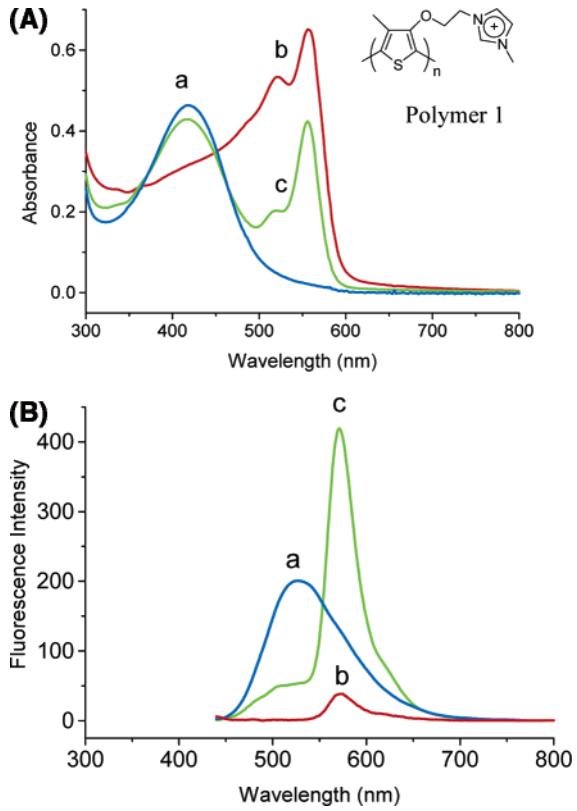

Figure 1. (A) Chemical structure of polymer 1 and UV−visible absorption spectra of (a) polymer 1/X1/Y1 triplex (perfect match), (b) polymer 1/[X1+Alexa Fluor (AF) 546] duplex, and (c) polymer 1/X1+AF546/Y1 triplex (perfect match) in water at 55 °C. (B) Fluorescence spectra, with excitation at 420 nm, of (a) polymer 1/X1/Y1 triplex (perfect match), (b) polymer 1/[X1+AF546] duplex, (c) polymer 1/X1+AF546/Y1 triplex (perfect match) in water at 55 °C.

3′-GTACTAACTTGGTAGGTGGT-5′) to the capture strand, triplex formation results in a helical conformation of the polythiophene leading to a yellow color ($\lambda_{max} = 421$ nm; Figure 1A, a) with fluorescence at 530 nm (Figure 1B, a). This helical structure of the polythiophene in the triplex form was confirmed previously by circular dichroism measurements.[10]

A new integrated DNA sensor was then designed by combining this polymeric transducer with capture probes labeled with a fluorophore (e.g., Alexa Fluor 546) in order to induce Förster resonance energy transfer (FRET). With this new transduction strategy, the stoichiometric duplex still gives a red color (Figure 1A, b) and quenched fluorescence (Figure 1B, b). When this duplex hybridizes with its complementary oligonucleotide, a new absorption feature appears at 420 nm, which is related to the formation of a triplex (see Figure 1A, c). Upon excitation at 420 nm, the fluorescence band at 530 nm of the resulting triplex (the donor in this FRET scheme) overlaps neatly with the absorption spectrum of the acceptor (Alexa Fluor 546, absorption peaks at 516 and 556 nm in Figure 1A, c), which then emits at longer wavelengths (emission maximum at 572 nm; see Figure 1B, c). Upon addition of noncomplementary or mismatched DNA to the duplex, the complex remains in the red nonfluorescent form, preventing the FRET mechanism from occurring. Consequently, the fluorescence intensity measured (in the same experimental conditions) with the perfect complementary ss-DNA strand is always higher than that obtained with targets having two (i.e., Y2: 3′-GTACTAACTTCGAAGGTGGT-5′) mismatches or even one (i.e., Y3: 3′-GTACTAACTTCGTAGGTGGT-5′) mismatch.

More interestingly, as shown in Figure 2, starting with a large number of duplex probes (ca. 10^{10} copies), 30 copies of 20-mer target oligonucleotides are easily detected from a volume of 3 mL, and perfectly complementary targets can still be distinguished at such low concentrations from sequences having two or even one mismatch. The detection limit calculated from these data is five copies in 3 mL, or 3 zM, with a custom blue-LED fluorimeter, whereas a somewhat higher detection limit of 30 copies in 3 mL, or 18 zM, was obtained using a commercial (Varian Cary Eclipse) spectrofluorometer (see Supporting Information). Previous studies described an amplified quenching of the signal,[15−20] whereas our approach involves a "turn-on" signal amplification.[10,11,15] In the present case, the amplification of the signal does not come only from the large optical density of the polymer but could also come from a fast and efficient energy transfer[15−20] from the helical and well-structured polythiophenes to many neighboring chromophores, a process that can be described as "superlighting" or "fluorescence chain reaction" (FCR) (see Figure 3). Moreover, because of the small Stokes shift of the Alexa Fluor chromophore, it is possible that energy transfer occurs also between these aggregated chromophores.[19] To support these assumptions, dynamic light scattering measurements have clearly revealed the formation of nanoaggregates of duplexes ca. 80 nm in diameter, which are preserved upon hybridization. Moreover, it is worth noting that this photonic amplification effect is not observed for unlabeled ss-DNA probes, for which limits of detection in the attomolar range were reported.[13]

This novel signal amplification detection scheme also makes possible the direct detection of ds-DNA at ultralow concentration levels. Because the probe-to-target hybridization reaction is in competition with rehybridization of the ds-DNA, most previously reported direct DNA detection techniques rely on the availability of the target sequence as ss-DNA. In the case of our polythiophene transducer, previous studies[10,13] have shown that the presence of noncomplementary ds-DNA may lead to false positive signals since the polythiophene has a higher affinity toward ds-DNA compared to that of the ss-DNA probes. However, experimental conditions have been found that selectively enhance the recognition reaction between the DNA capture probe and the DNA target. Specifically, in pure water at 65 °C, all denaturated DNA material remains denaturated and hybridization essentially only occurs with labeled ss-DNA probes in the duplexes, promoted by the electrostatically bound cationic polythiophene transducer, which also serves as a localized counterion for the negative charges of the phosphate moieties.

The high selectivity provided by the FRET-enhanced detection scheme and this ability of the polythiophene transducer to promote hybridization in otherwise unfavorable conditions was

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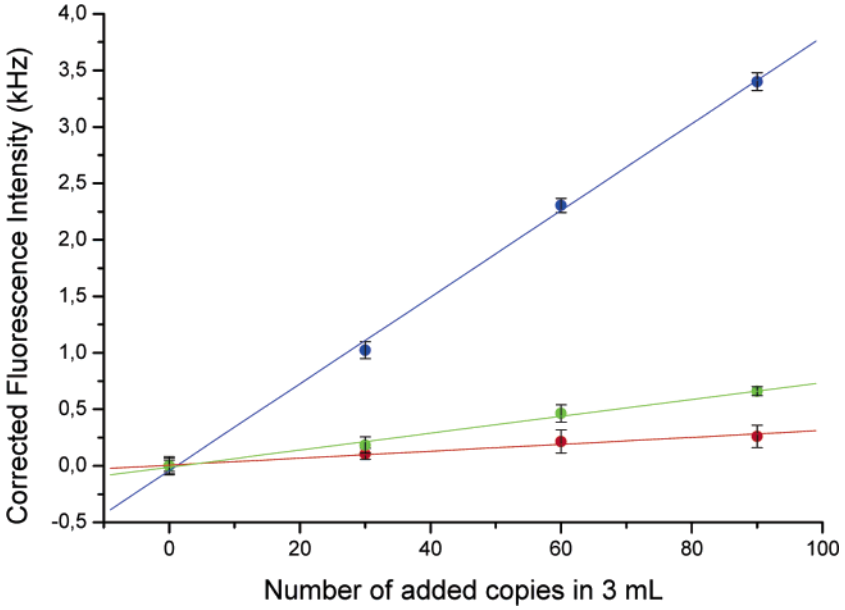

Figure 2. Corrected fluorescence intensity (after subtraction of the signal due to initial duplex) measured at 572 nm, with excitation at 420 nm in pure water at 55 °C, as a function of the number of 20-mer oligonucleotide target copies: (blue) polymer 1/X1+AF546 + Y1 (perfect match); (red) polymer 1/X1+AF546 + Y2 (two mismatches); (green) polymer 1/X1+AF546 + Y3 (one mismatch).

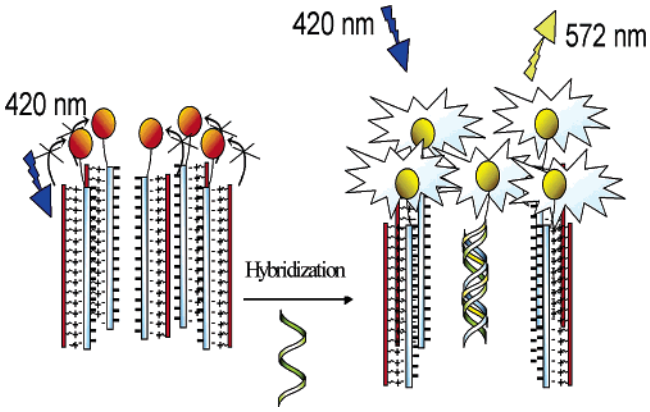

Figure 3. Schematic description of the proposed signal amplification detection mechanism based on the conformational change of cationic polythiophene and energy transfer for ultrasensitive, selective, and rapid DNA detection.

used to distinguish disease-associated single nucleotide polymorphisms (SNPs) in nonamplified human genomic DNA samples. For instance, Figure 4A shows calibration curves obtained by testing normal (i.e., wild type) human genomic DNA, with probe sequences complementary to the wild type (TAN 101: 5′-CCG GTG AGT ATC TGG-3′) and mutated (TAN 100: 5′-CCG GTG AAT ATC TGG-3′) sequences of the gene defective in the human genetic disease, hereditary tyrosinemia type I.[21,22] Similarly, Figure 4B shows curves obtained with mutated DNA (from ill patients) tested with the same two probes. Interestingly, heterozygote samples gave a fluorescence signal about half of the values obtained for the perfect match with homozygote samples. These data were obtained in 5 min and show that we can easily and rapidly distinguish wild-type DNA from mutated DNA; that is, we can detect a single nucleotide polymorphism (SNP) in the entire genome, without prior amplification or enrichment of the target and at concentration levels similar to those achieved with 20-mer oligonucleotides (Figure 2), that is, about five copies in the entire 3 mL sample volume. Other examples are provided in the Supporting Information.

Furthermore, although both 15-mer oligonucleotides used to probe tyrosinemia in the example above were designed to be unique in the human genome, there are numerous loci presenting only one mismatch with these probes, notably, at the extremities. However, no significant hybridization signal was detected from these nonspecific target sequences. We believe that very high stringency is obtained by hybridizing in water at 65 °C, where the only counterion available to neutralize the phosphate backbone of the nucleic acids is the cationic polythiophene transducer localized on the capture probe, therefore, permitting exquisite specificity. All these features suggest broad applicability of our new method to selectively detect few copies of target nucleic acid sequences in complex mixtures, without the need for nucleic acid amplification. To the best of our knowledge and according to a recent review,[23] this combination of sensitivity, selectivity, and simplicity cannot be reached by any other available technique.

Conclusion

We have developed an integrated molecular system which combines a specific receptor, an optical transducer, and an amplification mechanism. This novel method is based on different electrostatic interactions between a cationic polythiophene (i.e., polymer 1) and ss-DNA or ds-DNA, and the efficient energy transfer between the resulting triplex (complexation between the cationic polythiophene and ds-DNA) and neighboring fluorophores attached to ss-DNA probes. This impressive methodology leads to the specific detection of few copies of nucleic acids in 5 min in water. The sensitivity and specificity of this new approach should, therefore, lead to simple, rapid, and cost-effective diagnostic tools without any PCR

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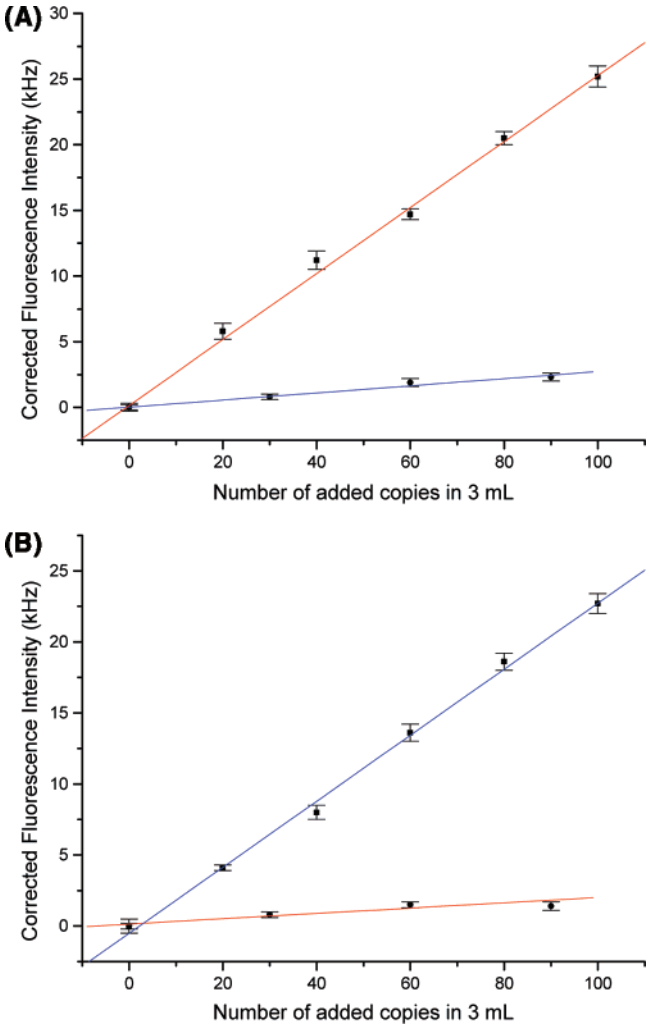

Figure 4. (A) Corrected fluorescence intensity (after subtraction of the signal due to initial duplex) measured at 572 nm, with excitation at 420 nm in pure water at 65 °C, as a function of the number of genomic DNA copies: (red) TAN 101 + wild-type genomic DNA (perfect match); (blue) TAN 100 + wild-type genomic DNA (one mismatch). (B) Corrected fluorescence intensity (after subtraction of the signal due to initial duplex) measured at 572 nm, with excitation at 420 nm, as a function of the number of genomic DNA copies: (blue) TAN 100 + mutated genomic DNA (perfect match); (red) TAN 101 + mutated genomic DNA (one mismatch).

amplification steps. In addition, this polymeric system shows all of the necessary features to be easily adapted for multiparametric detection on solid supports.

Experimental Section

Materials. Polymer 1 was synthesized according to our previously published work.[10,13] On the basis of size-exclusion chromatography measurements calibrated with monodisperse polyvinylpyridinium samples, this polymer has a number-average molecular weight of 11 000 with a polydispersity index of 2.0.[24] Labeled and unlabeled oligonucleotides were purchased from Integrated DNA Technologies, Inc. For studies on 20-mer oligonucleotides, the probe (X1) and targets (Y1, Y2, and Y3) were derived from probes designed for the detection of *Candida* yeast species.[10] For the studies involving human genomic DNA, the hereditary disease called *hereditary tyrosinemia* was chosen as a target because of its severity and importance in the Quebec region.[21] We first focused our efforts on the IVS12 splice mutation, mostly because it is well documented and very frequent. For the detection of tyrosinemia type I IVS12+5 G→A splice mutation,[21,22] the 15-mer capture probe sequence complementary to the mutated sequence in the genome was (TAN 100) 5′-CCG GTG AAT ATC TGG-3′, and the capture probe complementary to wild-type DNA was (TAN 101) 5′-CCG GTG AGT ATC TGG-3′. Alexa Fluor 546 was attached at the 5′-end of the oligonucleotide probes. For the detection of a specific sequence in the entire human genome to be quantitatively valid, it is very important to verify that this sequence is unique. The probes TAN 100 and TAN 101 were thus tested against the human genome, using BLAST and FASTA calculations and the GenBank database. The TAN 100 and TAN 101 probe sequences were found to be unique to the IVS12 mutation. Despite this uniqueness, it should be noted that several sites displaying a single nucleotide difference were found. All oligonucleotides solutions were diluted with sterilized water, and all dilutions and solution handling were performed in plasticware.

Extraction and Purification of DNA from Blood. Human genomic DNA used in Figure 4 was extracted from patient blood as previously described[22] and stored frozen at −20 °C until use and aliquoted to the desired concentrations.

General Procedure for Optical Measurements. UV−visible absorption spectra (Figure 1A) were taken using a Hewlett-Packard (model 8452A) spectrophotometer. Fluorescence spectra (Figure 1B) were recorded on a Varian Cary Eclipse spectrofluorometer, while the fluorescence calibration curves (Figures 2 and 4) were obtained on a custom portable fluorometer described elsewhere[13] and modified for measurement of Alexa Fluor emission at 572 nm. In all cases, excitation was made at 420 nm, and the fluorescence data points on the calibration curves come from the average of five optical measurements at 572 nm. Each optical measurement is obtained by the integration of the fluorescence signal over a period of 10 s. For all optical measurements, 3 mL quartz cells with an optical path length of 1.0 cm were utilized. The limit of detection is calculated as 3 times the standard deviation of the optical measurements for the blank signal, divided by the slope of the calibration curve. Duplexes were prepared by mixing stoichiometric quantities of the polymer and of the oligonucleotide capture probes to give a concentration of 2.14 μM (stock solution). The resulting complex was then diluted to the desired concentrations. Hybridization experiments were carried out at 55 °C for the 20-mer oligonucleotides and at 65 °C for the detection of tyrosinemia SNPs. For the tyrosinemia studies, the samples were first denaturated at 100 °C.

Dynamic Light Scattering Measurements. Dynamic light scattering measurements were made on a Malvern Zetasizer Model 3000 HSA. An aqueous solution of the polymer-labeled probe complex ("duplex") at a concentration of 2.67×10^{-7} M (or 1.61×10^{11} copies/μL) was analyzed. The resulting particle size distributions were narrow (fwhm < 20 nm) and obtained several times with good precision ($\chi^2 < 2$).

Acknowledgment. This work was supported by the Chemical, Biological, Radiological, and Nuclear Research and Technology Initiative (CRTI), the Natural Sciences and Engineering Research Council of Canada (NSERC), and the Canadian Institutes for Health Research (CIHR). K.D. acknowledges also NSERC for a scholarship. The authors thank Dr. Farid Bensabaa (ICPET, NRC, Ottawa, Canada) for assistance during dynamic light scattering measurements, as well as Professors G. D. Scholes (University of Toronto), R. D. J. Miller (University of Toronto), and J. L. Brédas (Georgia Tech) for valuable discussions about energy transfer in conjugated systems.

Supporting Information Available: Additional detection examples using a commercial (Varian Cary Eclipse) spectrofluorometer or a custom blue-LED fluorimeter with different DNA targets. This material is available free of charge via the Internet at http://pubs.acs.org.

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