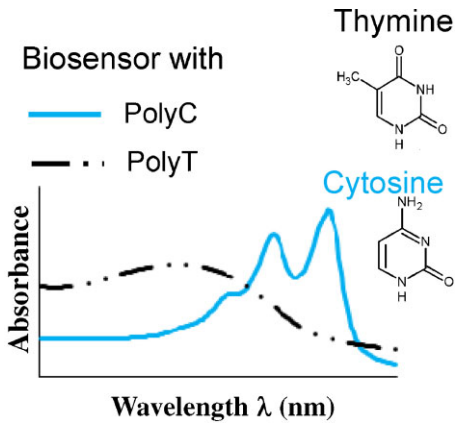
Impact of DNA Sequence and Oligonucleotide Length on a Polythiophene-Based Fluorescent DNA Biosensor[a]

Isabelle Charlebois, Catherine Gravel, Naoual Arrad, Maurice Boissinot, Michel G. Bergeron, Mario Leclerc*

DNA hybridization is a universal and specific mechanism for the recognition of biological targets. Some cationic polythiophene transducers sensitive to DNA structure have been previously utilized to detect such biomolecules. Further characterization of these systems indicates that both DNA sequence composition and length modulate the biosensor performance. It appears that different repeated sequence patterns cause different conformational changes of the polythiophene, from a more relaxed form to an extremely rigid one. A length difference between the DNA oligonucleotide probe and target has a detrimental effect on the fluorescent signal, but it can be attenuated by changing the sequence composition of the protruding target sequence. This demonstrates that the nature of DNA can be critical for hybridization-based detection systems.



1. Introduction

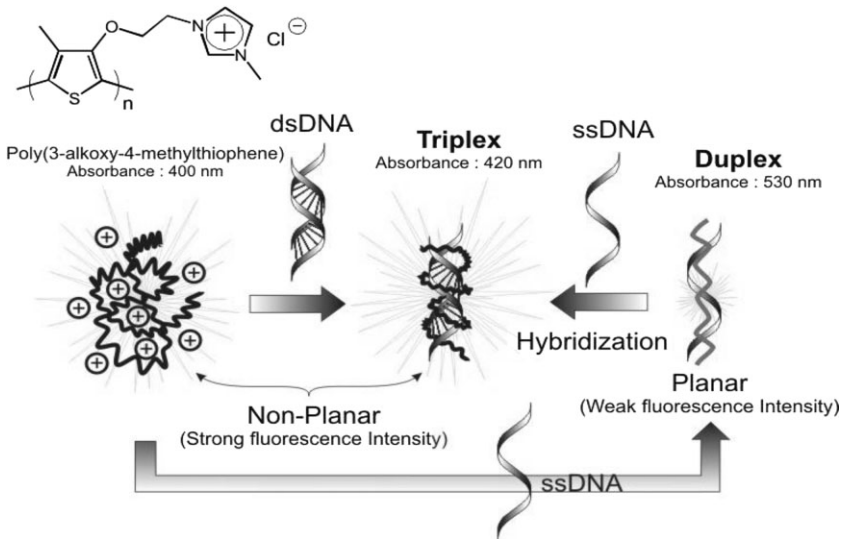
DNA hybridization is the cornerstone of many biomedical detection tests. Indeed, while DNA is universal in cellular life forms, the specific sequence of the nucleotide bases makes it unique. In addition, the formation of Watson-Crick base pairs through multiple hydrogen bonds and the double-stranded (ds) helical structure of DNA allow the identification of specific complementary sequence through hybridization. Many studies have been carried out to develop detection systems to generate a physically measurable signal from the recognition event of DNA hybridization.[1–5] Among them, chemical biosensors usually contain two basic components connected in series: 1) a biochemical recognition system, such as DNA hybridization, and 2) a physicochemical transducer to generate an analytically useful signal.[6]

Sensors based on luminescent conjugated polymers (LCPs) have been shown to be very sensitive to minor perturbations and possess remarkable electronic and optical properties making them advantageous over small-molecule-based sensors.[7–9] Moreover, any anionic

I. Charlebois, Dr. M. Boissinot, Prof. M. G. Bergeron
Centre de recherche du CHU de Québec, Département de Microbiologie-infectiologie et immunologie, Faculté de médecine, Université Laval, 2705 Laurier Blvd., Quebec City, QC, G1V 4G2, Canada
C. Gravel, N. Arrad, Prof. M. Leclerc
Département de chimie, Université Laval, Quebec City, QC, G1V 0A6, Canada
E-mail: Mario.Leclerc@chm.ulaval.ca

[a] **Supporting Information** is available from the Wiley Online Library or from the author.

DOI: 10.1002/mabi.201200469



Scheme 1. Schematic description of the hybridization detection system; poly(3-alkoxy-4-methylthiophene), the cationic biosensor, is electrostatically attracted by DNA and displays different optical properties whether it is mixed to single-stranded (ss) DNA (duplex) or double-stranded (ds) DNA (triplex). Free polymer adopts a non-planar conformation and is highly fluorescent while, in the duplex form, it adopts a planar conformation with very low fluorescence. Upon hybridization of the duplex with a complementary DNA strand (i.e. generating dsDNA), the polymer conformation switches form planar to non-planar and thus regains much of its fluorescence emission properties. Fluorescence difference between duplex and triplex allows DNA detection. Additionally, absorbance properties of the polythiophene indicates its conformation state; free polymer ($\lambda_{max} = 400$ nm), duplex ($\lambda_{max} = 530$ nm) and triplex ($\lambda_{max} = 420$ nm).

polyelectrolyte such as DNA can form strong electrostatic complexes with a cationic polyelectrolyte such as LCPs, thus generating neutral complexes (co-acervates) being known to form stable aggregates. Few years ago, our team reported a new cationic polythiophene derivative[10] allowing simple optical (colorimetric or fluorimetric) detection of DNA oligomers.[11] This cationic polymer displays optically measurable conformational change upon DNA hybridization (see description in Scheme 1). Its positively-charged substituents are electrostatically attracted by the negative charges of the DNA backbone creating a DNA-polymer complex. In contact with a single-stranded (ss) DNA, the polymer adopts a planar and almost non-fluorescent form called ''duplex''. Upon hybridization of the ssDNA to its complementary strand thus forming dsDNA, the polymer shifts to a non-planar conformation called ''triplex'', emitting a highly fluorescent signal. This system has proven to be very sensitive and specific by allowing the differentiation of as low as one mismatch on a 20-mer DNA strand.[11] Absorbance spectral properties are indicative of the polythiophene conformational state ($\lambda_{max} = 400$ nm for free polymer, $\lambda_{max} = 530$ nm for duplex and $\lambda_{max} = 420$ nm for triplex).

To have a better understanding of this biosensor system, it is important to further characterize its recognition component. In this work, it will be demonstrated that DNA sequence composition and length of the probe and target may have a direct impact on the fluorescence signal of the polythiophene biosensor upon hybridization. Results revealed that repeated patterns of sequence modulate the polymer conformation from a rigid and planar form to a more relaxed one, close to that one obtained with the free polymer. As they modulate the polymer conformation, sequence patterns can be used to enhance the fluorescence signal for the recognition of target longer than that of the probe.

2. Experimental Section

2.1. Materials

Poly(3-alkoxy-4-methylthiophene) was synthesized as previously described in the literature.[10,11] Sodium chloride (NaCl) and hydrogenated Triton X-100 were obtained from Aldrich. Water was purified through a Thermo Scientific Barnstead NANOpure water purification system. Oligonucleotides were purchased at Integrated DNA technologies (IDT Inc., Coralville, IA). The biologically relevant sequences are listed in Table 1 and were designed from genome sequence of *cap*C or *lef* gene from *Bacillus anthracis* or *tef*1 from *Candida albicans*. Oligonucleotides with repeated sequence pattern are described in details in Table S1 in the Supporting Information.

2.2. Absorbance Measurements

All absorbance experiments were carried out in 100 μL of 0.1 M NaCl solution in a quartz cell (Starna Cells Inc., Atascadero, CA, catalog number: 26.100LHS-Q-10/15) at 50 °C using a UV-HP8452 spectrophotometer (HP, Palo Alto, CA). In a typical experiment, a 0.1 M NaCl solution was placed in a quartz cell and heated at 50 °C in the spectrophotometer for 5 min prior to measurement (used as blank for all following measurements). The polymer was then added to the solution to obtain a final 3-alkoxy-4-methylthiophene monomer equivalent of 7.3×10^{-5} M and incubated in the spectrophotometer for 5 min. The oligomer DNA probe was added to obtain the same monomeric concentration and incubated in the spectrophotometer for 5 min for duplex formation. Target oligomer DNA was finally added at the same concentration than the probe and absorbance spectra were then recorded at different times. Notably, after each addition, the cell content was mixed by pipetting.

Table 1. List of the oligonucleotides corresponding to biologically relevant sequences used in this study.

ID	Gene	Length	Sequence
L226	*lef*	25	AATTAAAAGCTTCCGGTGCATAAAG
L226RC	*lef*	25	CTTTATGCACCGGAAGCTTTTAATT
C234	*cap*C	25	GGCAACGCTAATTACAGGTATTTGT
C234RC	*cap*C	25	ACAAATACCTGTAATTAGCGTTGCC
C271	*cap*C	25	CTGATTCTTGTTATCATGTTATGCC
C271RC	*cap*C	25	GGCATAACAGGATAACAATAATCAA
T406	*tef*1	20	CATGATTGAACCATCCACCA
T406RC	*tef*1	20	TGGTGGATGGTTCAATCATG
T406RC40	*tef*1	40	CTCACAATTCCACACAACATTGGTGGATGGTTCAATCATG
T406GAA	T406RC + GAA repeat	40	GAAGAAGAAGAAGAAGAAGATGGTGGATGGTTCAATCATG

2.3. Fluorescence Measurements

Fluorescence experiments were carried out at 50 °C, using quartz cells (Starna Cells Inc., Atascadero, CA, catalog number: 3-Q-10-GL14-C) on a Cary Eclipse spectrofluorimeter (Varian Inc., Palo Alto, CA) with the Photomultiplier Tube set at maximum (1000). Fluorescence spectra were recorded with an excitation wavelength of 420 nm. All concentrations were monomeric of either polythiophene polymer or DNA oligonucleotide. The duplex (polymer + probe complex) were made by mixing stoichiometric (in charges) quantities to obtain 1.2×10^{-4} M of polymer and fluorescence measurements were done at 2×10^{-7} M of polymer in 3 mL of a 0.1 M NaCl + 0.0003 M hydrogenated Triton X-100 solution. In a typical experiment, the salt-Triton solution was delivered in a quartz cell and heated at 50 °C in the spectrofluorimeter for 5 min (used as blank for duplex fluorescence analysis). After each addition, the content of the cell was mixed by pipetting. The duplex was then added in the heated solution and incubated in the spectrofluorimeter for 5 min prior to measurement of the fluorescence spectra (used as blank for triplex fluorescence analysis). After that, a target DNA was added and the fluorescent signal was then measured at different times.

3. Results and Discussion

3.1. Effect of DNA Sequences

As described in Scheme 1, the fluorescent hybridization detection signal was obtained upon conformational change of the polymeric transducer from a planar (duplex) form to a non-planar (triplex) one. Results presented in Figure 1 show the fluorescence signal of the polythiophene biosensor, measured after 30 min of triplex formation, using various oligonucleotide sequences as probe and target (detailed in Table 1). Those results revealed that, upon hybridization, some DNA sequences emitted higher fluorescence signals than others. For example, the maximum fluorescence signal of C234 with C234RC was more than twice the signal obtained for L226 with L226RC. In addition, for all the cases presented in this study, results in absorbance measurements showed that the difference in fluorescence signal was reflected by a faster switch from 530 nm (λ_{max} of the duplex) to 420 nm (λ_{max} of the triplex) absorbance peak for the "high fluorescence signal" sequences compared to the "weak fluorescence signal" ones (data not shown). Those results indicated that the speed of the conformational change, from the planar to the nonplanar one, mediates the fluorescence intensity obtained upon hybridization. Furthermore, the rapidity of the polythiophene conformational change was affected by the sequence of the DNA components.

Interestingly, the order of addition of the DNA oligomer to the reaction mixture also has an impact on the fluorescence signal intensity measured upon hybridization.

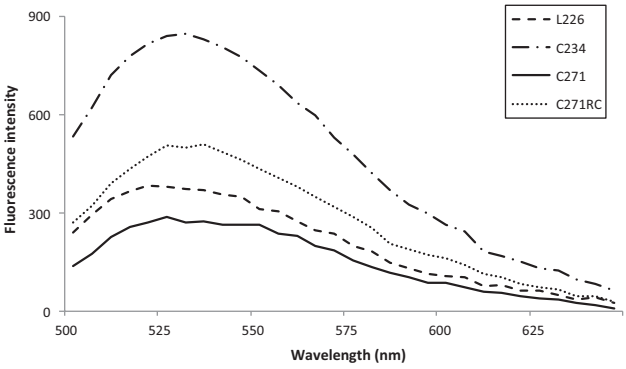


Figure 1. Fluorescence spectra of triplexes measured after 30 min of hybridization reaction. For each curve, the fluorescence spectrum of the duplex was subtracted. All of the DNA probes and targets were 25-base oligonucleotides and are listed in Table 1. The name of each curve corresponds to the ID of the probe used for duplex formation. They were hybridized with their complementary target: L226 with L226RC, C234 with C234RC and C271 with C271RC or vice versa.

For example (see Figure 1), when C271 was used as a probe to form the duplex and was then hybridized with C271RC, the maximum fluorescence signal measured was half of that obtained when C271RC was used as a probe and C271 as the target. Since the final product of both hybridizations was the same, it was hypothesized that the difference in fluorescence emission signal upon hybridization can be explained by the structure adopted by the duplex. Lacking the intermolecular hydrogen bonding which stabilize the dsDNA structure, ssDNA displays various structures directly mediated by its sequence[12] and those variations may reflect onto the polythiophene biosensor structure in the duplex. As a result, some ssDNA sequences could generate a more rigid planarization of the polymer than others. The conformational change upon hybridization should be slowed by a more rigid duplex, hence reducing the fluorescence signal emitted.

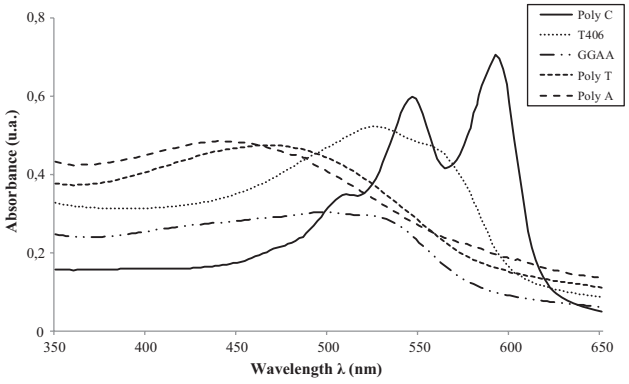


Figure 2. Absorbance UV–vis spectra of polymer mixed with ssDNA oligonucleotides of 20 bases displaying different absorbance peaks: PolyC with λ_{max} at 600 nm, biological sequence from *tef1* of *C. albicans* (T406) with λ_{max} at 530 nm, GGAA repeat with λ_{max} at 500 nm, polyT with λ_{max} at 460 nm and polyA with λ_{max} 435 nm.

3.2. Effect of Repeated Pattern of Sequences

To investigate the impact of DNA sequence on the polymer conformation, different sequence patterns were tested. They go from the simple poly A, C, G or T to alternate pattern like "GTA"; from low to high GC contents; from only purine to purine/pyrimidine alternation (a list of the 52 tested sequences is available in Table S1 in the Supporting Information). These DNA oligomers have been mainly tested in duplex for absorbance measurements as it allows visualization of the polymer conformation. We found that those oligomers displayed various absorbance peaks ranged from 600 nm, the extremely planar form, to 435 nm, very close to the triplex absorbance peak at 420 nm. One duplex, made with polyG, even showed a maximum absorbance peak of 420 nm, indicating a twisted conformation. However, these can be explained by the known properties of polyG sequences to form G-quadruplexes via Hoogsteen base pairing.[13] The results obtained with polyG were not further considered in the evaluation of the impact of the sequence onto polymer conformation. Besides polyG, the absorbance results obtained with the sequence with repeated patterns can be separated in 5 groups. One typical example of the absorbance spectra of each group is presented in Figure 2: PolyC with a maximum absorbance peak at 600 nm, T406 with a peak at 530 nm, GGAA repeating units with a peak at 500 nm, polyT with a peak at 462 nm and polyA with a peak at 435 nm. T406 was included as a marker of the result obtained for biologically relevant sequences with all four bases. One of the more interesting results was the absorbance measurement obtained with polyC and polyT. Their impact on the polymer conformation is very different even though the chemical structure of their nucleobases, Cytosine and Thymine, is similar. In particular, polyC duplex gave a conformation so rigid and uniform that three vibronic peaks are clearly visible. On the contrary, polyT duplex gave a maximum absorbance peak of 462 nm indicating a less planar conformation, close to conformation of the triplex (420 nm). Absorbance changes upon duplex hybridization with its complementary sequence were also tested for all the sequences with repeated pattern. The results have been separated in 4 different categories based on the appearance of an absorbance peak at 420 nm and the speed of change in absorbance (see Supporting Information, Table S1). For half of those sequences, the polymer did not achieve the full triplex conformation at 420 nm. Interestingly, this conformation was achieved when the DNA hybridization occurred prior to contact with the polymer. Those results, along with the results obtained with biological sequences, indicate that the conformation adopted by the polymer with the ssDNA probe (duplex) is modulated by the DNA sequence and it is critical for the hybridization detection. As simple as DNA hybridization seems, all sequences are not equally efficient for use as recognition component with chemical sensors sensitive to change in the DNA structure.

3.3. Effect of Length of DNA Probe

The effect of the length of probe was also investigated. The optimal length of probe was determined to be from 8 to 35 bases as the duplex is not fluorescent and the whole reaction switch from duplex to triplex is completed within 10 min or less. Polymer mixed with probe of fewer than 8 bases did not take a planar conformation, therefore the fluorescence emission of the duplex is very high and no fluorescence increase was measurable upon hybridization. On the other hand, absorbance measurement of the switch from duplex to triplex with probes of 40 to 60 bases showed

that the reaction was very slow and still not complete even after 30 min. Consequently, the fluorescence signal of triplex formed with long probes was very weak, almost non-existent. Length of the probe has an impact on the structure adopted by the DNA which impacts on the polymer conformation and properties. Very short probe did not adopt an organized structure and the polymer remains non-planar while it is the contrary for long probes. Again those results showed the importance of the DNA probe in the whole detection system, not only by its recognition capacity but by its structure.

3.4. Effect of Length and Sequence of DNA Target

Investigation tests showed that length of the DNA target also has also an impact on the detection signal. Indeed, the use of target longer than the probe has a detrimental effect on the fluorescence signal obtained upon hybridization. As shown on Figure 3, while the triplex formation made with the 20mer probe T406 and target T406RC gave a high fluorescence signal, triplex formation with the same probe hybridized to a target twice its length, T406RC40, generated almost no fluorescence. It is believed that the polymer could be attracted by the higher negative charges of the long DNA target, creating a mix of duplexes made with short and long ssDNA, and prevented or delayed hybridization. Part of the polymer must remain planar on the ssDNA protruding sequence and prevent the polymer conformational change upon hybridization. Interestingly, when DNA hybridization is performed prior to contact with the polymer, the displayed conformation is similar to that one obtained with equal length probe and target, i.e. non-planar, highly fluorescent with λ_{max} of 420 nm. In the latter case, the double helical conformation of the DNA is probably more stable and dominates the ssDNA protruding sequence structure. It was supposed that, since DNA sequence has an effect on the conformation adopted by the polymer in duplex and that repeated sequence patterns displayed a non-planar polymer conformation while in duplex, this knowledge could be used to design a long target that would generate high fluorescence signal upon hybridization. In that particular target, the protruding sequence has a repeated pattern that does not prevent the polymer to switch from planar to non-planar conformation. It is shown in Figure 3, where hybridization of a 20mer (T406) duplex with a 40 base target containing a protruding sequence of GAA (T406GAA) gave a fluorescence signal equivalent to that one obtained with the 20mer target T406RC. This result indicates that it is possible to improve fluorescence signal by selecting appropriate sequences.

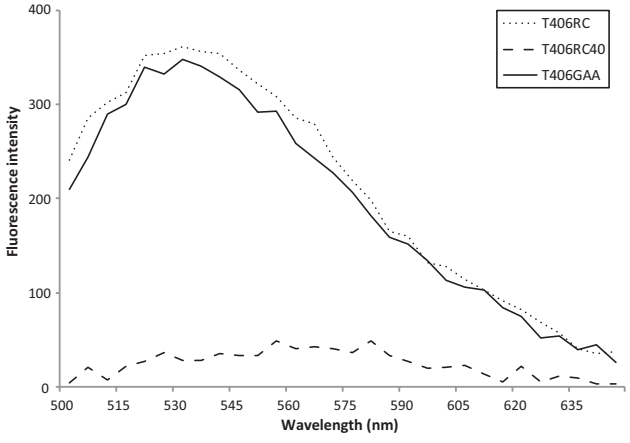


Figure 3. Fluorescence intensity of the triplexes (polymer/probe/target) obtained for the probe T406 with: **1**: T406RC, its 20 base complementary target, **2**: T406RC40, a 40 base complementary target where 20 bases of biological sequence were added to C406RC and **3**: T406GAA a 40 base complementary target where 20 bases of repeated GAA sequence were added to T406RC.

4. Conclusion

Many biosensor systems utilize the recognition mechanism of DNA hybridization as it is very specific, relatively simple and present in all cellular life forms. However, it is also a very sensitive mechanism that could be affected by different parameters. In the present case of a polythiophene biosensor sensitive to DNA hybridization, it was demonstrated that the DNA sequence and its length affect the biosensor conformation and, as such, its detection properties. Particularly, sequences with repeated pattern had given various conformations modifying the luminescent properties of the duplexes. With that knowledge, it was possible to modulate the signal by changing the sequence and eliminate the impact of target length. Although these experiments have been done with a specific biosensor system, the parameters identified here may apply for other DNA hybridization sensors. Base pair recognition by DNA hybridization may look as a simple process, but a better knowledge of its component and their interaction could be critical for any biosensing system.

Acknowledgements: I. Charlebois and C. Gravel contributed equally to this work. This work was supported by Defence Research and Development Canada Centre for Security Science Chemical, Biological, Radiological/Nuclear, and Explosives Research and Technology Initiative (CRTI-03-0005RD, CRTI-06-0186RD and CRTI-06-0187TD). The authors also wish to thank Dr. Ahmed Najari for suggestions, discussion and assistance in the design of Scheme 1.

Received: December 18, 2012; Revised: February 6, 2013; Published online: March 19, 2013; DOI: 10.1002/mabi.201200469

Keywords: affinity chromism; conjugated polymers; DNA; oligonucleotides; polythiophenes

[1] X. Liu, Q. Fan, W. Huang, *Biosens. Bioelectron.* **2011**, *26*, 2154.
[2] J. Lei, H. Ju, *Chem. Soc. Rev.* **2012**, *41*, 2122.
[3] L. Shao, Y. Gao, F. Yan, *Sensors* **2011**, *11*, 11736.
[4] L. Wang, P. C. H. Li, *Anal. Chim. Acta* **2011**, *687*, 12.
[5] E. G. Hvastkovs, D. A. Buttry, *Analyst* **2010**, *135*, 1817.
[6] V. Tsouti, C. Boutopoulos, I. Zergioti, S. Chatzandroulis, *Biosens. Bioelectron.* **2011**, *27*, 1.
[7] T. M. Swager, *Acc. Chem. Res.* **1998**, *31*, 201.
[8] L. H. Chen, D. W. McBranch, H. L. Wang, R. Helgeson, F. Wudl, D. G. Whitten, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12287.
[9] S. W. Thomas, G. D. Joly, T. M. Swager, *Chem. Rev.* **2007**, *107*, 1339.
[10] H.-A. Ho, M. Boissinot, M. G. Bergeron, G. Corbeil, K. Doré, D. Boudreau, M. Leclerc, *Angew. Chem. Int. Ed.* **2002**, *41*, 1548.
[11] K. Doré, S. Dubus, H.-A. Ho, I. Lévesque, M. Brunette, G. Corbeil, M. Boissinot, G. Boivin, M. G. Bergeron, D. Boudreau, M. Leclerc, *J. Am. Chem. Soc.* **2004**, *126*, 4240.
[12] J. Isaksson, S. Acharya, J. Barman, P. Cheruku, J. Chattopadhyaya, *Biochemistry* **2004**, *43*, 15996.
[13] M. Adrian, B. Heddi, A. T. Phan, *Methods* **2012**, *57*, 11.