SNP detection for hereditary neuralgic amyotrophy alleles by target-assembled tandem oligonucleotide systems based on excimers

Salah M. Bensaber, Ibrahim A. Mrema, Mousa I. Jaeda and Abdul M. Gbaj *
Department of Medicinal Chemistry, Faculty of Pharmacy, University of Tripoli, Tripoli, Libya
*Correspondance to abdulgbaj1@hotmail.com

Abstract: Hereditary Neuralgic Amyotrophy (HNA), also known as Brachial Plexus Neuropathy, is a rare hereditary disease that is characterized by recurrent episodes of severe arm and shoulder pain accompanied by muscle weakness, nerve deterioration, and sensory impairment of the nerves in the arm. Linkage studies have shown that HNA is an autosomal dominant disorder caused by a genetic defect localized to the long arm of the seventeenth chromosome between the 24th and 25th band (17q24-17q25). Detection of specific target nucleic acid sequences is commonly achieved by hybridization of fluorescently labeled oligonucleotide probe(s) to a complementary target sequence. We report the first use of excimer-based split-probes for detection of the wild type and mutant alleles of Hereditary Neuralgic Amyotrophy. A tandem 15-mer split DNA oligonu-cleotide probe system was designed that allows detection of the complementary target DNA sequence. This excimer-based fluorescence detector system operates by means of a contiguous hybridization of two oligonucleotide excimer split-probes to a complementary target nucleic acid target. Each probe oligonucleotide is chemically modified at one of its termini by a potential eximer-forming partner, each of which is fluorescently silent at the wavelength of detection. Under conditions that ensure correct three-dimensional assembly, the chemical moieties on suitable photoexcitation form an eximer that fluoresces with a large Stokes shift (in this case 130 nm). The excimer system was able to differentiate wild type and mutated SNP (835 A→G) alleles, based on fluorescence emission spectra indicating promise for future applications in genetic testing and molecular diagnostics.

Keywords: Excimer, pyrene, Hereditary Neuralgic Amyotrophy

Introduction

Hereditary neuralgic amyotrophy (HNA) is a neuralgic disorder that is characterized by nerve damage and muscle atrophy, preceded by severe pain (1, 2). It is caused by a mutation in the SEPT9 gene (17q25). HNA is an episodic disorder; it is characterized by episodes generally lasting 1-6 weeks. During an episode, the nerves of the

brachial plexus are targeted by the body as antigens, and the body's immune system begins to degenerate the nerves of the brachial plexus. The exact order or location of the nerve degeneration cannot be predicted before an episode. Other areas of the nervous system that have been affected are the phrenic nerves and the recurrent laryngeal. As the nerves lose function, the muscles associated with those nerves begin to atrophy. In brachial plexus degeneration, atrophy may occur in the deltoid muscles. In phrenic nerve degeneration, the diaphragm may be affected. In this case, breathing can be impaired due to a lack of muscle control of the diaphragm. If the recurrent larangyl nerve is targeted, the pharynx will begin to atrophy and voice function may be lost (3, 4).

The rapidly increasing speed the DNA sequencing in the recent years has begun to make accessible much information on the underlying genetic causes of inherited and acquired diseases. It is becoming clear that in the future, methods for rapidly screening patient-derived samples for known disease-related sequences will be an important tool in the arsenal for prevention and treatment of disease. Of the many techniques which are being developed for DNA sequence identification, solution-based fluores-cence hybridization methods offer a number of advantages which make them the subject of much recent study (5, 6). Among the advantages of solution-based fluorescence methods is the fact that they may require no further manipulation such as gel or blot analysis after being exposed to the target DNA. In addition, they can in some cases be used in real-time analysis of sequences being amplified in the polymerase chain reaction (PCR) (7, 8). Pyrene is a simple hydrocarbon aromatic molecule, having an advantage of adaptability for various chemical modifications, and gives monomer and excimer emissions depending on its concentration (9). It was reported that the synthetic macrocyclic molecules, in which two pyrene moieties were placed parallel to each other in close proximity. Owing to the spatial arrangement of the pyrene rings, the macrocycles emit only excimer fluorescence. Since the formation of pyrene excimer strongly rests upon the extent of π-plane overlaps, the main excimer emission of the macrocycles showed the care for our DNA molecular design. This finding encouraged us to apply such molecular design technique to develop novel oligonucleotide probes (10-12).

Detection of specific target nucleic acid sequences is commonly achieved by hybridization of a fluorescently labeled probe oligonucleotide a complementary to a target, but this usually has very large background fluorescence signals. It is possible to decreased the background by using two short oligonucleotide probes that are complementary to neighbouring sites of the same DNA target, and can form a pyrene excimer (Fig. 1). Excitation of one of the pyrene partners, forms an *exci*ted state di*mer* (excimer) that emits at long wavelength (Stokes shifts >100 nm) (13-15). Thus, the target assembles its own detector from components which are non-fluorescent at the detection wavelength, which gives a massive improvement in terms of the reduced background.

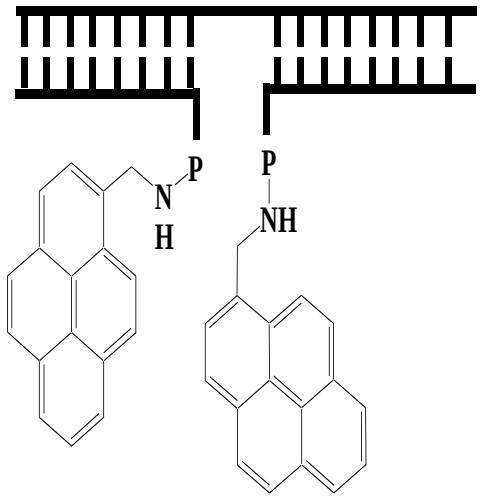

Figure 1: Representation of the split-probe excimer system to be studied

Materials and methods

Nature of the target.

The excimer construction studied used normal DNA base/ sugar structures in both target and probes. The 30-mer DNA target sequence selected, dTCAAACAGCGGATCACCGCAGACCTGCTGT, is non self-complementary sequence and free from possible secondary structure complications. Furthermore, the possibility of hairpin-stem formation is negligible. The 30-mer target is a part of Homo sapiens septin 9 (SEPT9), transcript variant X6, mRNA chromosome sequence (http://www.ncbi.nlm.nih.gov/nuccore/XM_006721644.1) (Figure 2).

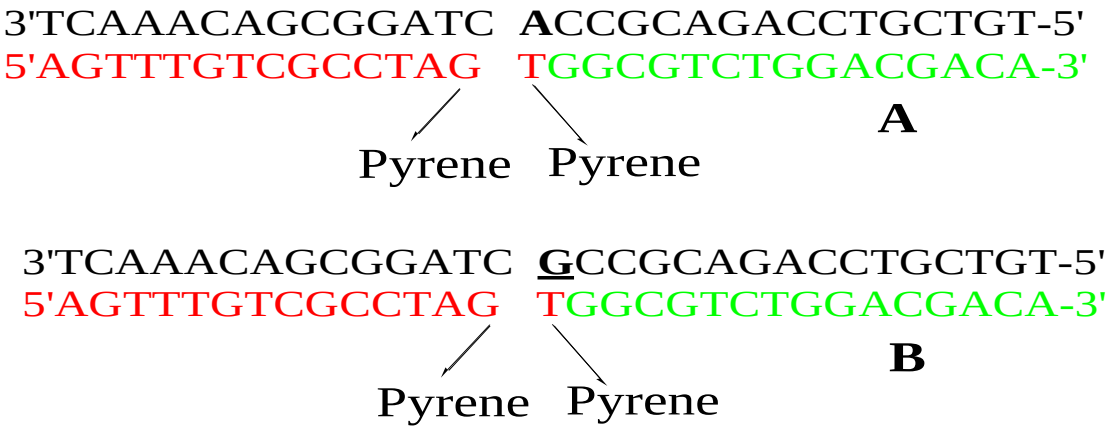

Figure 2: Homo *sapiens septin* 9 (SEPT9), transcript variant X6, mRNA chromosome sequence, the green and red oligonucleotides bases are the complementary strands of the target as pyrene attached probes. (**A**) is the wild type target and (**B**) the SNP target, the underline **G** is the muation.

Reagents and materials

Reagents of the highest quality available were purchased from the suppliers indicated. Aldrich Chemical Company Ltd, Poole**:** Cetyltrimethylammonium bromide; 1-pyrenemethylamine; triphenylphosphine; dipyridyldisulfide; dimethylaminopyridine; triethylamine; lithium perchlorate; dimethylformamide; trifluoroacetic acid; magnesium chloride. Sigma Aldrich, UK: DNA oligo probes, DNA targets (parent and mismatches). BDH Ltd., Liverpool**:** Acetone; tris(hydroxylmethyl)methylamine; methanol; ethanol; sodium chloride.

Preparation of the probes

Oligonucleotide conjugates with excimer partners at the 3'- or 5'-phosphate groups were prepared, purified and characterised as described (16-18).

HPLC

Reverse phase HPLC purification of oligonucleotides was performed using a HPLC Holochrome 302 (Gilson) chromatograph equipped with a C18 column (Vydac™, particle size 10 µ, inner diameter 10 mm, length 250 mm, pore size 300 Å) with elution using an increasing gradient (0 - 40%) of acetonitrile in water (fraction detection at 260 nm).

Fluorescence spectroscopy: UV-Visible absorption spectra were measured at 20 °C on a Cary-Varian 1E UV-Visible spectrophotometer with a Peltier-thermostatted cuvette holder and Cary 1E operating system/2 (version 3) and the CARY1 software. Quantification of the oligonucleotide components used millimolar extinction coefficients (ε_{260}). Fluorescence excitation and emission spectra were recorded on a Varian Eclipse Fluorescence Spectrophotometer with a Peltier-thermostatted cuvette holder. For comparative purposes the standard component concentration of tested samples in the cuvette was 2.5 µM. Spectra were recorded in Tris buffer (10 mM Tris, 0.1M NaCl, at pH 8.5) containing various percentages of TFE or other co-solvents (0 - 80%) using a 2 mL thermostatted, 4-sided, quartz cuvette. Excitation wavelengths for both monomer and excimer emission were optimised in each experiment and ranged from 340 to 350 nm. In most cases slit widths were set from 5 to 10 nm both for excitation and emission spectra, depending on the intensity of emission. "Automatic shutter-on" regime was used to minimize photo degradation of compounds in the cuvette. All spectra were corrected for buffer, TFE and/ or naphthalene emission as appropriate at the specific temperature and wavelength. In some cases spectra were scaled to the pyrene LES emission at 380 nm of the pyrene-bearing probe to facilitate visualisation of changes in the ratio between the pyrene LES and the excimer band. The ratio between the emission intensity of the excimer band at 480 nm and the LES band at 380 nm (I_E/I_M) was also calculated for each spectrum.

Duplex formation- synthetic oligonucleotides: The complex was formed by sequential addition of aliquots (5 µL) of the components from stock solutions (1 x 10^{-3} M) to Tris buffer (10 mM Tris, pH 8.40, 100 mM NaCl) (2 mL) containing the appropriate amount of TFE (0-80%) at 10 ºC. Components (molar ratio 1:1:1) were added in the order: probes then target. The concentration of each component in the cuvette was 2.5 µM. On formation of the full complex, the system was allowed to equilibrate for 5 minutes at 10 ºC, and emission spectra then recorded at 3-minute intervals. The cuvette was then heated to 50 °C for 5 minutes and allowed to cool back to 10 ºC over 40 minutes. Emission spectra were then recorded at 3-minute intervals until excimer emission did not increase further (this usually occurred after 9 minutes). All spectra were baseline-corrected.

Duplex formation using synthetic oligonucleotide target (1 mL volume)

Excimer formation was induced by sequential addition of aliquots (2.5 µL) of 3'-pyrene oligonucleotide, 5'-pyrene-oligonucleotide, and finally target from the stock solutions (1 mM) to 100 µL Tris buffer containing a suitable amount of TFE (0-82.4%) at 5 °C, and the volume was made up to 1000 µL with Milli-Q water. The mole ratio of oligonucleotides used was 1 : 1 : 1 (the concentration of each component was 2.5 µM in the cuvette). Emission spectra were recorded after each component was added, using excitation wavelengths of maximum fluorescence intensity determined for the 3'-and 5'-pyrenyl oligonucleotide (340 nm) and for the full assembled excimer system (350 nm) using slitwidth 5 nm, to examine alterations in emission spectra resulting from complex construction. On construction of the full excimer complex, the system was allowed to equilibrate for 2-5 minutes at 5 °C or 20 °C, and emission spectra then recorded to monitor changes in excimer intensity. The excimer system in the cuvette was then heated from 5º C to 90 ºC at 5 ºC/min and allowed to cool back to 5 °C at a rate of 1 ºC/min. Emission spectra were recorded until excimer fluorescence intensity emission showed no further increase.

Results and discussion

Emission spectra confirmation:

Additional evidence of duplex formation comes from the emission spectra, as the one probe oligonucleotides did not excimer signal in the absence of the other complementary probe. Addition of the complementary probe to the probe oligonucleotide components (e.g. each bearing pyrene) caused a small red shift (3 - 5 nm) of the pyrene LES emission band, accompanied by its quenching. Then a new structureless band appeared at ~ 480 nm as shown in Figure 3. This band was characteristic of a pyrene excimer (19, 20). Similar quenching of the pyrene LES emission and appearance of an excimer emission was observed on formation of the tandem split-probe excimer systems of many research groups (19, 20). All studies reported that quenching of the LES emission band and the appearance of a pyrene excimer band on addition of the target strand to the probe components. These spectral changes suggested that some form of duplex formation had taken place.

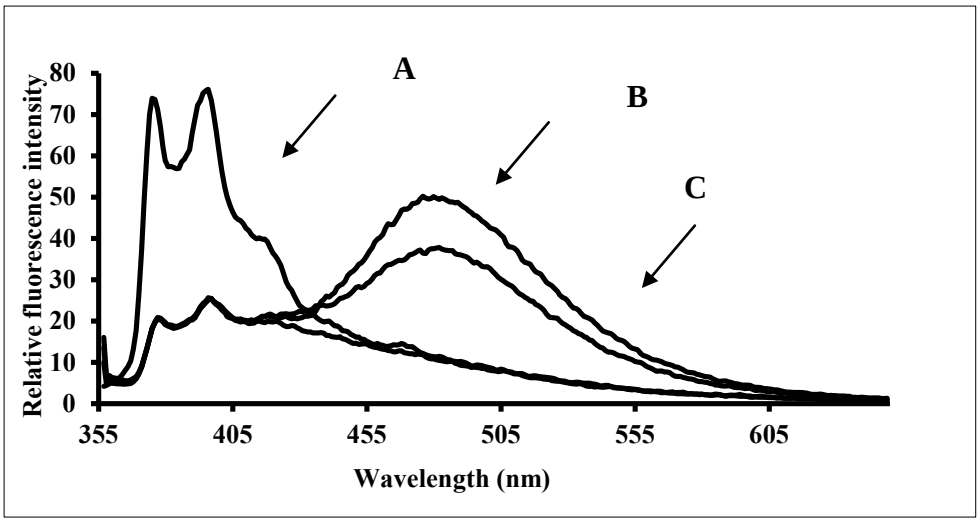

Figure 3: Excitation and emission spectra of the terminally located probe system (**A**) 3'-pyrenyl and 5'-pyrenyl probes **(B)** 3'-pyrenyl+ 5'-pyrenyl in 80% TFE/0.01 M Tris, 0.1 M NaCl, pH 8.4) at 5 ºC with wild type target and **(C)** 3'-pyrenyl+ 5'-pyrenyl in 80% TFE/0.01 M Tris, 0.1 M NaCl, pH 8.4) at 5 ºC with mutated type target (SNP). Component concentrations were 2.5 µM (equimolar).

Spectral changes observed on formation of the full system included, under the appropriate conditions (80% TFE/ Tris buffer, 0.01 M Tris, 0.1 M NaCl, pH 8.4 at 5 ºC) led to the appearance of a red-shifted structureless band at ~ 480 nm characteristic of excimer emission. Excimer emission is achievable due to interaction of the exci-partners on duplex formation if they are perfectly located close to each other.

The Hereditary Neuralgic Amyotrophy system was used to determine the effects of mismatches in the target gene, compared to the perfect target sequences, on excimer fluorescence at different temperatures. A single-base mismatch in DNA, where bases opposing one another in the duplex do not represent Watson-Crick A-T or G-C pairs, provides a perturbation in the base stack. The mismatched split-probe Hereditary Neuralgic Amyotrophy system studied in this paper demonstrated the characteristic red shift in LES emission indicating that duplex formation occurred on target addition. As for the normal Hereditary Neuralgic Amyotrophy system, the excimer emission at 480 nm was seen in the presence of the normal and mutated targets in a ratio of 1.4 to 1, respectively.

The advantages of using the spit probe excimer are that the pyrene labeling method is quite simple since it utilises a phosphoramidate derivative that can be incorporated at the end of a sequence after DNA synthesis, and has a long shelf-life (if kept in –20 °C), and is safe to handle, unlike radio-labeled probes. Once the probes have been prepared no further reactions are necessary. Assays can be performed in solution without the need for probe immobilisation, with no washing steps required to remove unbound probe. No cascade of reactions required for signal development, as for biotinylated

probes. Exciplexation does not require specialised instrumentation beyond a simple spectrofluorimeter, and does not require time-resolved measure-ements. Background fluorescence does not present a problem. The large Stocks shift of the pyrene excimer fluorescence (~140 nm) avoids spectral overlap and the interference that can occur with FRET-based strategies. The split-probe system could be tested against targets containing various mismatches and insertions to determine whether this system could be of use in detecting SNPs in genetic sequences, which might broaden the application of such a dual-probe strategy. These results of the excimer formation present additional evidence of tandem duplex formation, showing only one transition, consistent with the literature findings that duplex structures containing a backbone nick in one of the strands melt cooperatively[19,20].

Conclusion: The use of a simple fluorescent nucleoside method in detection of DNA sequence and point mutations by hybridisation in solution is described in this paper. Excimer based split-probe systems can signal the presence of a target DNA sequence. This study, supported by literature data, suggests that the tandem split-probe system, consisting of the target sequence and two probe oligonucleotides bearing the exci-partners can assemble correctly at the specified target so that the exci-partners end up side by side. Once the whole system (two probes and target) has assembled under suitable conditions, a red-shifted excimer emission is seen on irradiation at a suitable wavelength. The results of these studies suggest that in most situations the band at 480 nm can be attributed to pyrene-pyrene interaction for excimer and pyrene-naphthalene interaction for excimer emission. This excimer emission shows a very large Stokes shift (~ 140 nm) and in most cases an extremely small background. However, these attractive photo-chemical properties of pyrene also result in serious drawbacks, such as a considerable quenching of its fluorescence by the presence of oxygen, electron-donating and -accepting molecules that may exist in vivo, low fluorescence quantum yield in protic solvents and the relatively short absorption wavelengths of pyrene (21). The excimer-based split-probe system was a used for detection of wild type (WT) and SNP alleles of human Hereditary Neuralgic Amyotrophy using 15-mer probes, labelled with pyrene partners at the 5′ or 3′ termini, respectively, were evaluated. These results show that it is possible to use excimer probes for detection of Hereditary Neuralgic Amyotrophy SNPs, with promise for future applications in genetic testing and molecular diagnostics.

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