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Interaction of fluorescent dyes with DNA and spermine using fluorescence spectroscopy

*K. Gracie[a], W. E. Smith[a], P. Yip[b], J. U. Sutter[b], D. J. S. Birch[b], D. Graham[a], K. Faulds[a]**

[a]*Centre of Molecular Nanometrology, WestChem, Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, G1 1XL, UK*

[b]*Department of Physics, Photophysics Group, Centre for Molecular Nanometrology, Scottish Universities Physics Alliance, University of Strathclyde, John Anderson Building, 107 Rottenrow East, Glasgow, G4 0NG, UK*

Abstract

Oligonucleotides labelled with fluorescent dyes are widely used as probes for the identification of DNA sequences in detection methods using optical spectroscopies such as fluorescence and surface enhanced Raman scattering (SERS). Spermine is widely used in surface enhanced based assays as a charge reduction and aggregating agent as it interacts strongly with the phosphate backbone and has shown to enhance the signal of a labelled oligonucleotide. The fluorescence intensity of two commonly used labels, FAM and TAMRA, were compared when spermine was added under different experimental conditions. There was a marked difference upon conjugating the free dye to an oligonucleotide, when FAM was conjugated to an oligonucleotide there was around a six fold decrease in emission, compared to a six fold increase when TAMRA was conjugated to an oligonucleotide. Dye labelled single and double stranded DNA also behaved differently with double stranded DNA labelled with FAM being a much more efficient emitter in the mid pH range, however TAMRA becomes increasingly less efficient as the pH rises. Upon addition of the base spermine, signal enhancement from the FAM labelled oligonucleotide is observed. Increasing probe concentrations of TAMRA oligonucleotide above 0.5 µM led to signal reduction most likely through quenching, either by an interaction with guanine, or through self-quenching. By using different bases for comparison, spermine and triethylamine (TEA), different affects were observed in the measured fluorescence signals. When TEA was added to FAM, a reduction in the pH dependence of fluorescence was observed, which may be useful for mid pH range assays. With the drive to increase information content and decrease time and complexity of DNA assays it is likely that more assays will be carried out in complex media such as extracted DNA fragments and PCR product. This model study indicates that dye DNA and dye spermine interactions are dye specific and that extreme care with conditions is necessary particularly if it is intended to determine the concentrations of multiple analytes using probes labelled with different dyes.

Introduction

Fluorescent dyes are widely used in biological applications, for example in cell imaging,[1] investigating protein interactions[2] and for the detection of oligonucleotides.[3, 4] Fluorescence and SERS detection methods using oligonucleotides labelled with fluorescent dyes are widely used because of the high sensitivity and ease of detection.[5, 6] However, detection methods for molecular diagnostics are a very active area with strong drivers to produce faster, simpler and more informative assays. This can involve detection *in situ* in a matrix such as cell lysate, extracted DNA or PCR product. In all these cases there is likelihood that interactions between the fluorophore and the matrix can alter the intensity of the emission. This becomes even more critical if multiple targets are to be determined since more than one dye may well be used and it raises the question of how dye specific the interactions are. In this study we compare the effect on the emission from two commonly used fluorescent dyes, FAM and TAMRA, at different pH and concentrations in the presence and absence of spermine. We report two interactions that occur; one between the fluorescent dyes and DNA and the other between the fluorescent dyes and spermine. It has already been widely reported that there is a strong interaction between spermine and double stranded DNA,[7, 8] however we propose that there is also a direct interaction between the spermine and the fluorescent dye. Spermine addition has a specific relevance to SERS and enhanced fluorescence assays since it is often used as an aggregating agent[9, 10] but the dye-DNA interactions and fluorescence quenching effects observed are dye specific and of interest in both fluorescence and SERS based assays.

Fluorescence emitted from a fluorophore can be quenched by a nucleobase, in particular guanine.[11-13] Guanine acts as a fluorescence energy acceptor through a photoinduced electron transfer mechanism.[12, 14] This phenomenon has been used successfully to quantitatively detect specific DNA sequences,[15] protein mutations,[13] and single base alterations.[16] Guanine quenching has also facilitated numerous studies focussing on the hybridisation of complementary oligonucleotides.[13] Nazarenko *et al.* reported that upon hybridisation of complementary DNA sequences, there was only a decrease in the fluorescence intensity emitted by fluorescein when either d(CC)or d(GG) bases and the fluorophore were present at the 5' terminus of the sequence.[17] Therefore, the design of oligonucleotide sequences is important when using fluorescence detection in hybridisation assays for the detection of specific DNA sequences.[18] Although the quenching effect of guanine and other nucleobases can be used for the detection of DNA and other biomolecules, it can be considered a major drawback when carrying out fluorescence measurements whe re quenching of the fluorescence emitted by the fluorophore is undesirable.

Polyamines, such as spermine, belong to a large group of biogenic amines that are involved in many physiological functions such as cell growth and proliferation and they are also involved in the synthesis of DNA and RNA.[19] Generally, polyamines are low molecular weight aliphatic amines that are water soluble and

have a pKa of around 10. They are protonated at physiological pH meaning they are polycations, which explains why they have such strong interactions with polyanionic macromolecules such as DNA.[7,8] Spermine is reported to have a strong interaction with DNA. Basu *et al* studied the effect of spermine on the aggregation and melting temperatures of calf-thymus DNA. Results showed that spermine encourages DNA aggregation and can cause the melting temperature of the DNA to increase as the spermine concentration is increased.[20] Many molecular studies have been performed to determine the effects of polyamines on double stranded DNA. Models were proposed that showed an interaction between the protonated polyamine group and the negatively charged double stranded DNA focussing on the electrostatic interactions, however direct interactions between the two were discarded.[21,22] Further studies on polyamine analogues showed that in addition to the electrostatic effects between the polyamine and double stranded DNA, structural specificities were also a major component between the interaction of spermine and double stranded DNA.[23] X-Ray diffraction studies of spermine-DNA crystals showed that spermine was positioned along the major groove of double stranded DNA meaning that the spermine did come into contact with the bases.[24,25] However, it was still unclear whether these specific binding sites existed in solution samples of spermine-DNA complexes. NMR studies on DNA solutions were carried out which indicated that the spermine-DNA interactions were highly localised therefore, it is not purely a result of electrostatic interactions.[26,27] It was also perceived that the NMR model alone cannot fully explain the spermine-DNA interactions as it was still believed that structural specificities would play a major role.[28] Ruiz-Chica *et al* studied the interaction between double stranded DNA and spermine by FT-Raman spectroscopy to achieve a deeper understanding of the spermine-DNA interaction in solution.[29] They concluded that there were specific binding sites, between the spermine NH_3^+ groups and either the purine-N7 or thymine-O4 atoms, that existed and this was in addition to the electrostatic effects previously reported.

The results produced from this model study demonstrate that not only does spermine interact with DNA; it has a strong interaction with the fluorophores that may be attached to the DNA sequence. Two fluorophores were used, FAM and TAMRA. Fluorescence results were compared before and after the addition of spermine under different experimental conditions and they suggested that spermine has a significant effect on the fluorescence emitted by FAM. Results obtained from the addition of spermine to TAMRA labelled DNA were not consistent with FAM fluorescence results, indicating that the spermine interactions are dye specific. These results show that great consideration is needed when selecting fluorophores to aid DNA detection when using fluorescence and SERS-based assays.

Materials and Methods

Oligonucleotides

All sequences were purchased on a 0.2 µM scale with HPLC purification from Eurofins MWG, Ebersberg, Germany (Table 1).

Table 1 Modified oligonucleotides used in this study

Name	Sequence (5'-3')	5' Modifications
TAMRA labelled oligonucleotide	GGTTCATATAGTTATAATAA	TAMRA
FAM labelled oligonucleotide	GGTTCATATAGTTATAATAA	FAM
Complementary Sequence	TTATTATAACTATATGAACC	-

Reagents

Spermine hydrochloride was purchased from Sigma Aldrich (Dorset, UK) and a stock solution of 0.1 M was prepared and diluted when required. Three different solutions were used for the pH studies performed. 250 mM trisodium citrate was prepared and pH was adjusted to 2.9 using HCl. 10 mM phosphate buffered saline, pH 6.9, was prepared by dissolving one tablet (Sigma Aldrich) in 200 mL distilled water. Finally, 1 M NaOH was prepared and pH was measured to be 13.2. The distilled water used to dilute spermine and oligonucleotides had a pH of 5.2.

Fluorescent Measurements

Reaction mixtures were made up to a final volume of 450 µL consisting of fluorophore labelled oligonucleotide (100 µL, 1 µM), either distilled water or one of the three pH adjusted solutions for pH studies (300 µL) and spermine hydrochloride (50 µL, 0.1 M). For double stranded DNA studies, 100 µL of each DNA sequence (Table 1) was added to 200 µL of 0.3 M phosphate buffered saline and underwent DNA hybridisation at 90 °C for 10 minutes followed by 10 °C for 10 minutes. The reaction mixture was added to disposable 1.5 mL PMMA semi-micro cuvettes. Measurements were recorded using a Horiba Scientific Fluorolog 3-22, which comprised of an Ushio UXL 450S-O 450 W Xenon short arc lamp as an excitation source, a 200-900 nm double grating excitation and emission monochromators and a R928 Hamamatsu photomultiplier tube. When measuring the FAM fluorescence emission the excitation wavelength was set to 494 nm with a scanning range 500-560 nm and when measuring TAMRA emission the excitation wavelength was set to 560 nm and the scanning range was 570-630 nm. The integration time was set to 0.1 sec and the slits widths were 5 nm for both excitation and emission. For each experiment, three replicate samples were prepared and each replicate was measured five times.

Results and Discussion

Fluorescent labelling is widely used in molecular diagnostics to facilitate the detection of DNA sequences coding for particular diseases. Specific DNA sequences are modified with a fluorescent dye and the response of the fluorophore measured using either fluorescence[30] or techniques such as surface enhanced Raman scattering (SERS).[31] The development of assays for multiple targets is of increasing importance as even more informative assays are sought and in some formats this involves the comparison of emission or scattering from more than one dye. However, fluorescent dyes are susceptible to interactions with the nucleobases in a DNA sequence,[32] to quenching, and to conditions such as pH and concentration. In addition, spermine has been used extensively in studies involving surface enhanced fluorescence (SEF)[9, 33] and surface enhanced Raman scattering (SERS).[10, 34] Spermine (Figure 1c) is used to reduce the negative charge on the surface of a nanoparticle and on the DNA allowing the DNA sequence to come into close proximity to the nanoparticle and if required to aid nanoparticle aggregation. To characterise the effect of changing conditions and of adding spermine, fluorescence from two commonly used fluorescent dyes are compared at different pHs and concentrations in the presence and absence of the bases spermine and triethylamine.

The two fluorophores used were FAM and TAMRA (Figure 1a, b) modified with the same DNA sequence to eliminate the issue of sequence specific affects.

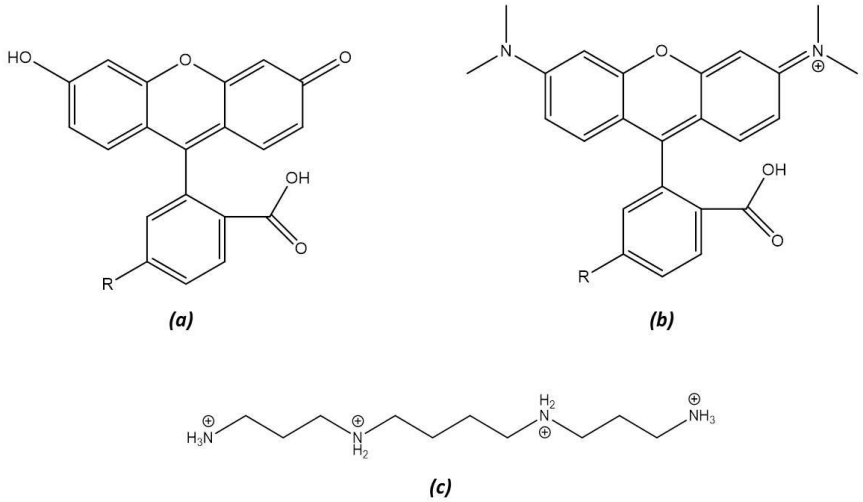

Figure 1 Structures of the two fluorophores and spermine used in this study. (a) Structure of FAM and (b) structure of TAMRA. In both cases R = NHS ester when the fluorophore is attached to an oligonucleotide and R = isothiocyanate when measurements were performed on the *free* dye. (c) Structure of spermine.

To determine the effect of attaching an oligonucleotide sequence to the fluorescent dye and of adding spermine on the fluorescence emission, the fluorescence from 1 µM solutions of the dye FAM isothiocyanate (FAM-ITC) and TAMRA isothiocyanate (TAMRA-ITC) and FAM labelled oligonucleotide and TAMRA labelled oligonucleotide was measured before and after a solution of 0.1 M spermine was added (Figure 2). No pH change was observed following spermine addition and the pH was 5.2 throughout. FAM-ITC fluoresced more strongly than FAM labelled oligonucleotide but the opposite result occurred with TAMRA. On addition of spermine there was an increase in fluorescence intensity of more than six fold with the FAM-ITC and FAM labelled oligonucleotide (Figure 2b) but with TAMRA-ITC and TAMRA labelled oligonucleotide there was a smaller decrease in fluorescence (Figure 2d).

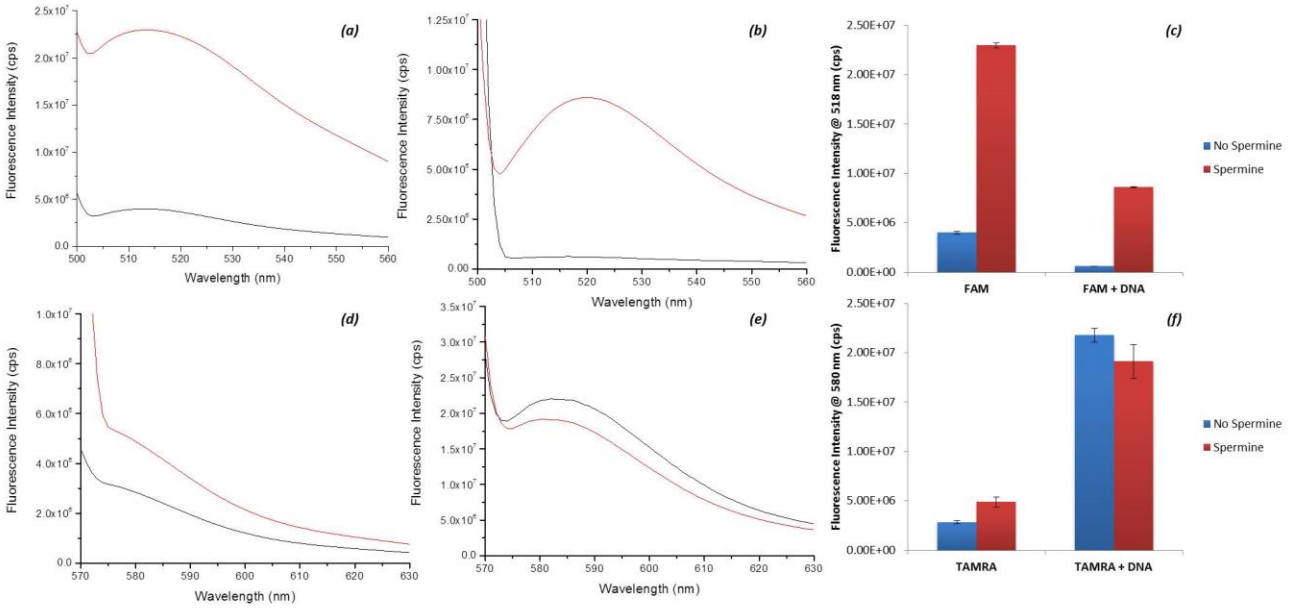

Figure 2 Results obtained when comparing the fluorescence emitted when spermine is added to *free* fluorescent dye or to dye-modified oligonucleotides. In the fluorescence spectra, the red spectra are those observed when spermine is added and the black spectra are those where spermine is absent. (a) Fluorescence spectra of FAM-ITC before and after spermine addition, (b) fluorescence spectra of a FAM-modified oligonucleotide before and after spermine addition, (c) fluorescence intensity comparison of FAM at 518 nm, (d) fluorescence spectra of TAMRA-ITC before and after spermine addition, (e) fluorescence spectra of a TAMRA-modified oligonucleotide before and after spermine addition and (f) fluorescence intensity comparison of TAMRA at 580 nm. 3 replicate samples were analysed 5 times. Error bars represent ±1 standard deviation.

The relationship between concentration and intensity over the range 1 µM to 1 nM for FAM labelled oligonucleotides is approximately linear except at the highest concentration. However, the concentration dependence for TAMRA oligonucleotide shows a drop in intensity at the highest concentration. This suggests that some aggregation of the DNA bases may be occurring in the case of TAMRA aided by the positively

charged amine in the zwitterion. TAMRA is not particularly susceptible to fluorescence quenching especially at low concentrations,[12, 14] but interaction with a DNA base on the oligonucleotide when an aggregate is formed would enable energy transfer and account for the drop in intensity (Figure 3b).

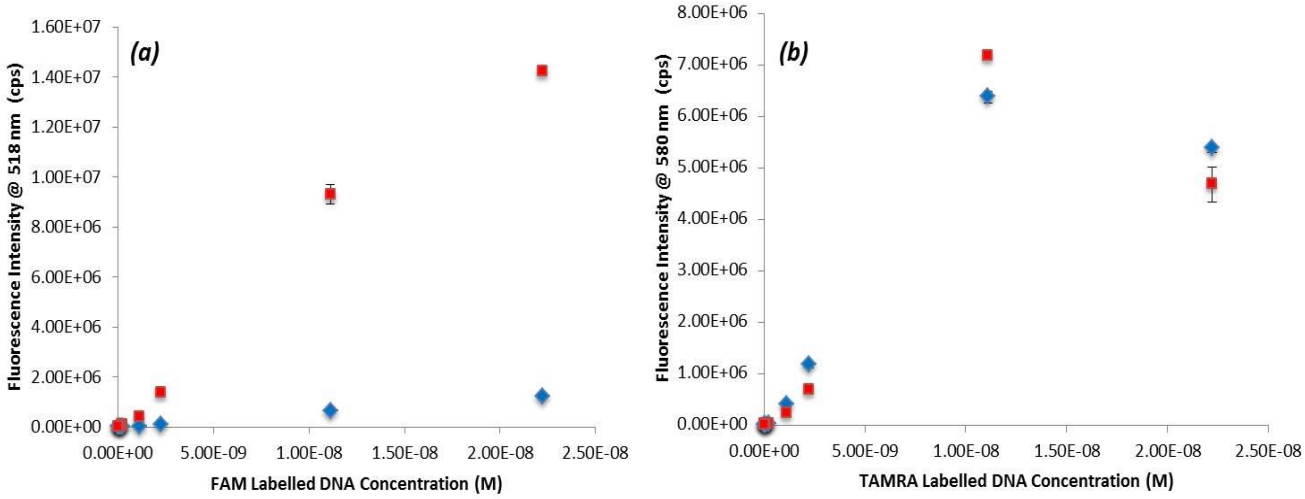

Figure 3 Fluorescence intensity at varying fluorescent dye modified DNA concentrations. (a) Fluorescence intensity at 518 nm when varying the concentration of FAM-labelled oligonucleotide, in the presence (red) and absence (blue) of spermine. (b) Fluorescence intensity at 580 nm when varying the concentration of TAMRA-labelled oligonucleotide, in the presence (red) and absence (blue) of spermine. 3 replicate samples were analysed 5 times each, error bars represent ± 1 standard deviation.

For FAM labelled oligonucleotides there is a clear relationship between fluorescence intensity and spermine concentration (Figure 4a-b) but for TAMRA oligonucleotide there is little change in fluorescence (Figure 4 c-d) as would be expected since spermine appears to have a much smaller effect on the fluorescence emission from TAMRA, which is in good agreement with results shown in Figure 2f. Therefore, spermine is shown to have a stronger effect on the fluorescence intensity emitted by FAM than TAMRA, emphasising that these are dye specific interactions.

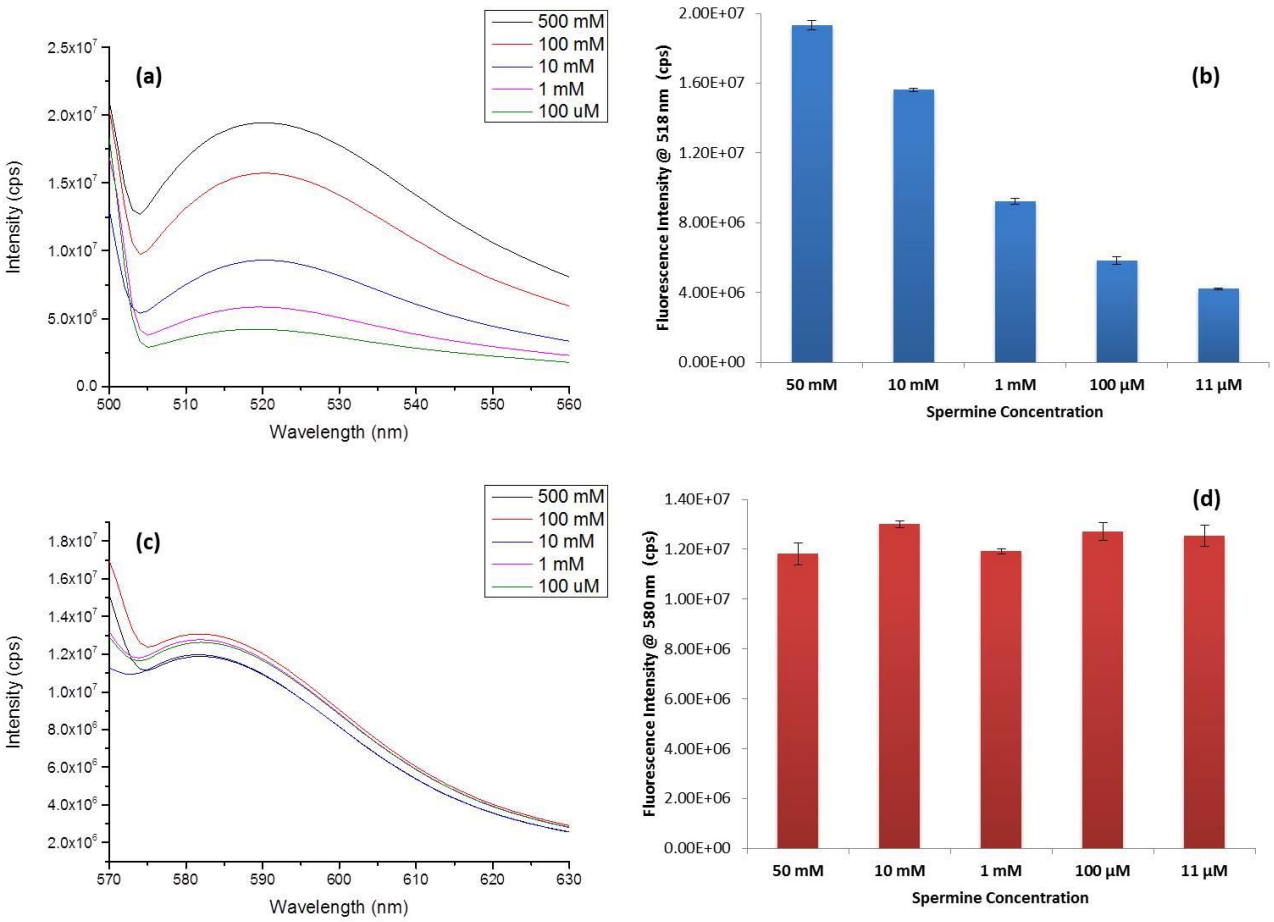

Figure 4 Results obtained when varying concentrations of spermine were added to the solution of fluorescent dye labelled oligonucleotides. (a) Fluorescence spectra obtained when decreasing the concentration of spermine that was added to a 1 µM solution of FAM-labelled oligonucleotide. (b) Comparison of fluorescence intensities at 518 nm over the concentration range of spermine added. (c) Fluorescence spectra obtained when decreasing the concentration of spermine that was added to a 1 µM solution of TAMRA-labelled oligonucleotide. (d) Comparison of fluorescence intensities at 580 nm over the concentration range of spermine added. 3 replicate samples were analysed 5 time and the error bars represent ± 1 standard deviation.

Fluorescence measurements of FAM and TAMRA were recorded at pH 2.9, 5.2, 6.9, 13.2. There was no additional change in pH upon addition of spermine. With increasing pH, the fluorescence intensity of FAM increases and the spermine attributed enhancement is present at all pHs. However the effect is much greater at pH 5.2. In contrast, the fluorescence intensity of TAMRA oligonucleotide varies less with pH as reported previously, but there is a significant increase at pH 6.9 (Figure 5c-d).[35, 36] There is a smaller enhancement due to spermine at pH 6.9 but at other pHs there is a small decrease.

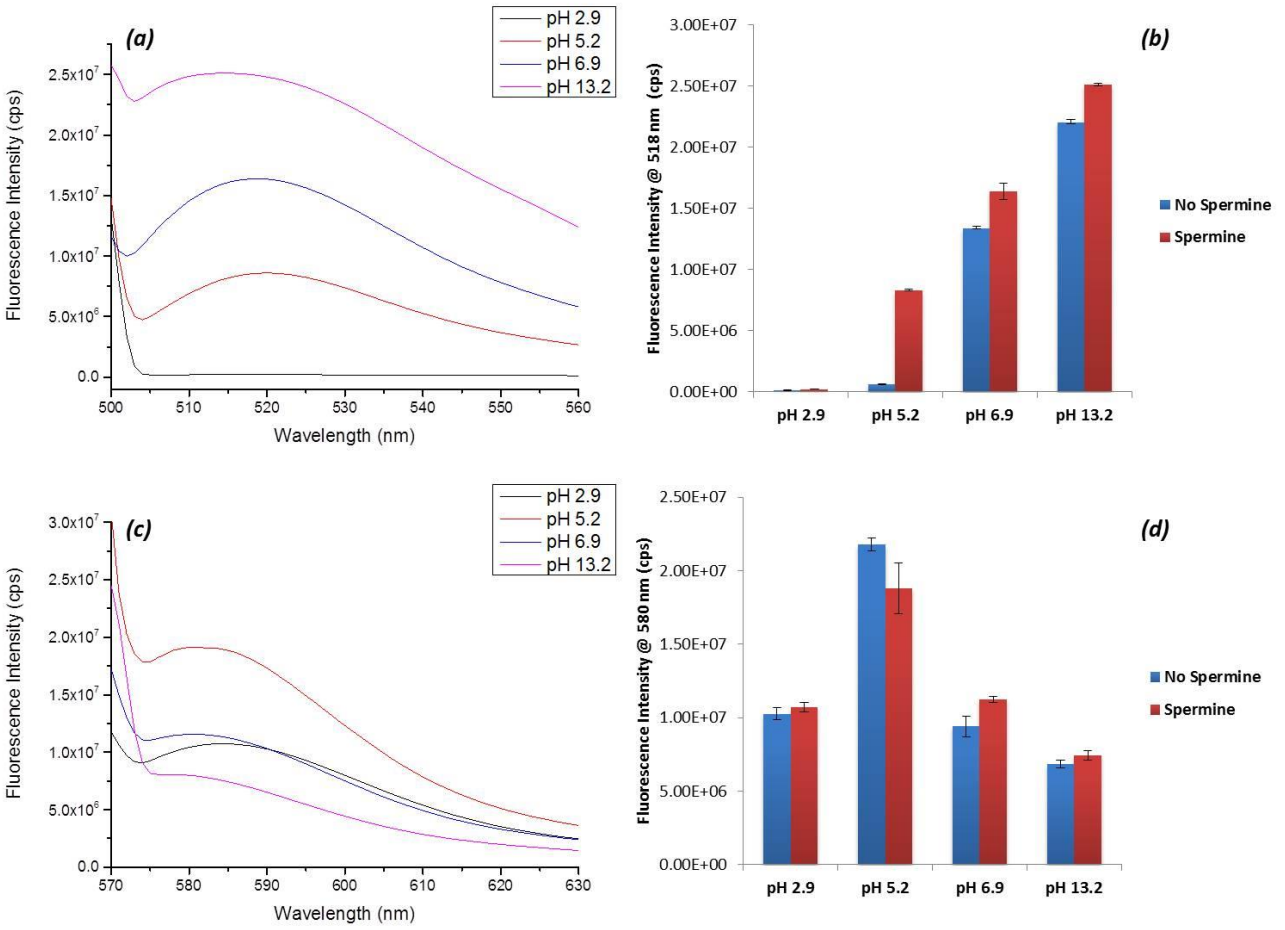

Figure 5 Fluorescence measurements obtained after varying the pH of the solution. (a) Fluorescence spectra of FAM-labelled oligonucleotides when the pH was altered. (b) Fluorescence intensity analysis at 518 nm, (c) fluorescence spectra of TAMRA-labelled oligonucleotides when the pH was altered and (d) fluorescence intensity at 580 nm. 3 replicate samples were analysed 5 times and the error bars represent ± 1 standard deviation.

The efficiency of emission from FAM and TAMRA is dependent on the dipole across the molecule and changes in the substituents on the ring will affect this as shown by the data in Figure 2 where the covalent attachment to an oligonucleotide sequence increases FAM efficiency and decreases TAMRA efficiency. The carboxylic acid group of FAM has a pKa of 3.9 (Figure 1) and the phenolic group a pKa of 6.5 and so at pH 5.2 FAM will exist in solution in equilibrium between the lactone and ionic forms (Figure 6a-b), where the lactone form gives very weak fluorescence.[37, 38] Positively charged spermine would be expected to bind across the molecule forming an acid base complex thus altering the equilibrium in favour of the ionic form.

Except in strongly alkaline conditions, TAMRA exists in equilibrium between its protonated and zwitterionic forms (Figure 6c, d) and its non-fluorescent lactone form (Figure 6e). The pKa of the carboxylic acid is very

low (pKa~3) so that across the pH range used, TAMRA will be present mainly in the zwitterionic form with some of the protonated form at the lowest pH.[39] The spectra show changes in peak position and intensity as the equilibrium changes with maximum emission at pH 6.9 where the zwitterion form should dominate. Covalent attachment to an oligonucleotide causes a big increase in intensity (Figure 2) presumably by stabilising the zwitterionic form and increasing the dipole. An acid base pair will form with spermine but the effect on the dipole will be more limited and more complex due to the positive charge on TAMRA.

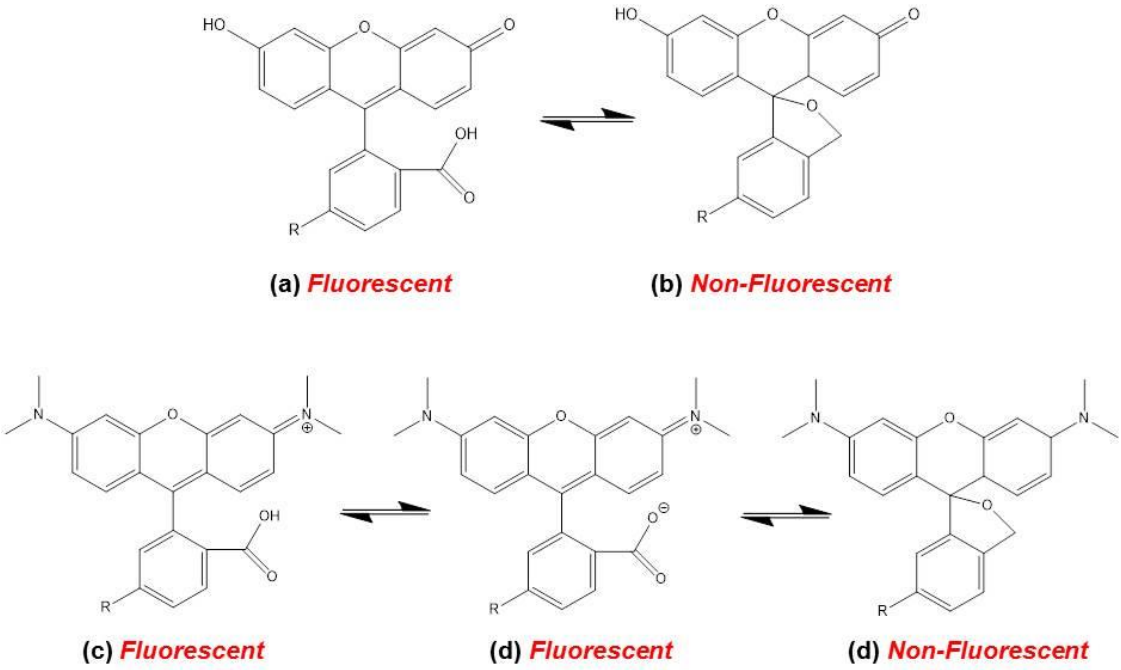

Figure 6 Structures of the fluorescent dyes at varying pH. (a) Ring-open form of FAM that is fluorescent and increases in the presence at increasing pH. At higher pH the phenolic OH will also ionise (b) Ring-closed lactone form of FAM present at acidic pH and is non-fluorescent. (c) Protonated form of TAMRA and (d) TAMRA zwitterion, both present at neutral and alkaline pH and fluorescent. (e) Lactone form of TAMRA that is non-fluorescent and exists at extremely acidic pH.

Fluorescence measurements were recorded before and after the addition of spermine to a solution of FAM or TAMRA labelled double stranded DNA (Figure 7a). The same spermine concentration study that was performed using single stranded fluorescent dye labelled DNA was repeated, however double stranded DNA labelled with FAM or TAMRA was used (Figure 7b). When adding spermine to fluorescent dye labelled double stranded DNA, there is no difference in emission between the two dyes FAM and TAMRA, furthermore when varying the spermine concentration the emission of the fluorescent dyes does not change. This is due to spermine having a stronger interaction with the double stranded DNA,[40] and in this situation it is the spermine-DNA interactions that dominate, i.e. the interaction between the fluorescent dyes and spermine is minimal resulting in little change in fluorescence emission, regardless of the fluorescent dye present.

The pH dependence of the fluorescence of both dyes in the double stranded form is markedly different. The FAM intensity at pH 2.9 was again low but at pH 5.2 and above it was nearly constant and greater than in the single stranded form (Figure 7c) even in the presence of spermine. It is most likely that the ionic form of the dye is favoured due to steric hindrance. In contrast, the fluorescence intensity of TAMRA was lower than in single stranded form and surprisingly decreased significantly with increasing pH with no peak in intensity at pH 6.9 (Fig 7d). In strongly alkaline conditions, the hydrogen bonds between the base pairs of the double helix will break resulting in two separate DNA strands.[41] Therefore at pH 13.2 the DNA attached to the fluorescent dye may no longer be in duplex form and the fluorescence intensity should be similar to that of single stranded DNA at pH 13.2. However at all pHs the fluorescence is lower than at the equivalent pH for the single stranded case, suggesting that the covalent attachment between the fluorescent dye and the oligonucleotide causes a change in dipole which reduces emission efficiency. The lack of a peak in intensity at pHs where the zwitterion would be expected to form suggests that the oligonucleotide sequence covalently bound to the fluorescent dye prevents true zwitterion formation.

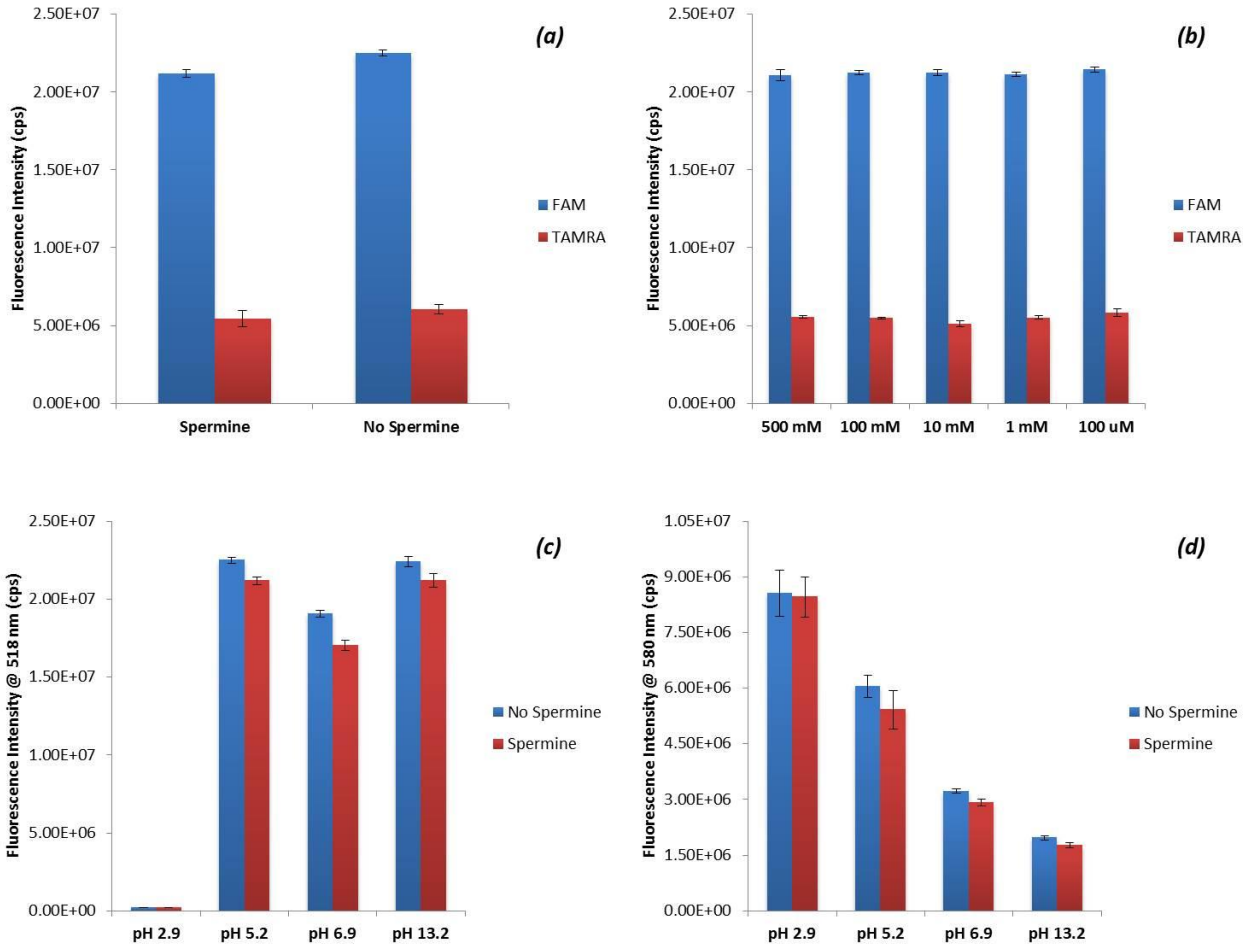

Figure 7 Fluorescence intensity comparisons obtained when fluorescent dyes were attached to double stranded DNA. (a) Fluorescence intensity at 518 nm (FAM) and 580 nm (TAMRA) before and after spermine addition. (b) Fluorescence intensity at 518 nm (FAM) and 580 nm (TAMRA) when concentration of spermine was decreased from 500 mM to 100 µM. (c) Fluorescence peak intensities at 518 nm comparing the change in FAM fluorescence with changing pH. (d) Fluorescence peak intensities at 580 nm comparing the change in TAMRA fluorescence with changing pH. For each experiment, 3 replicate samples were analysed 5 times and the error bars represent ± 1 standard deviation.

For comparison with the effect of spermine, the pH dependence experiments were repeated using a simple non-nucleophilic base, triethylamine (TEA), and both single and double stranded DNA (Figure 8). With single stranded FAM labelled oligonucleotide, maximum fluorescence was obtained at pH 5.2 and above, a result that is similar to that found for the double stranded product (Figure 7c). Perhaps not surprisingly for the double stranded FAM oligonucleotide, the base had little effect (Figure 8a). The TEA is present in excess and may form an acid base pair with both the carboxylic and phenolic groups by pH 5.2 whereas when spermine is present, it is already bound across the molecule may not affect the phenolic group.

For double stranded TAMRA labelled DNA the fluorescence decreases as pH increases and there is a much greater reduction in intensity at every pH than with spermine (Figure 8d). This is a surprising result since TEA

should not act as a TAMRA quencher. The most likely explanation is that the excess base is interacting with part of the Xanthine structure, probably the electron rich oxygen in the bridge, to alter the electronic structure and reduce the dipole. This may also be part of the reason it is so efficient at increasing the fluorescence efficiency of FAM. The fact that one effect is enhancement and the other reduction of fluorescence is reasonable since the efficiency of emission depends on the dipole and this will be altered differently in the two fluorophores.

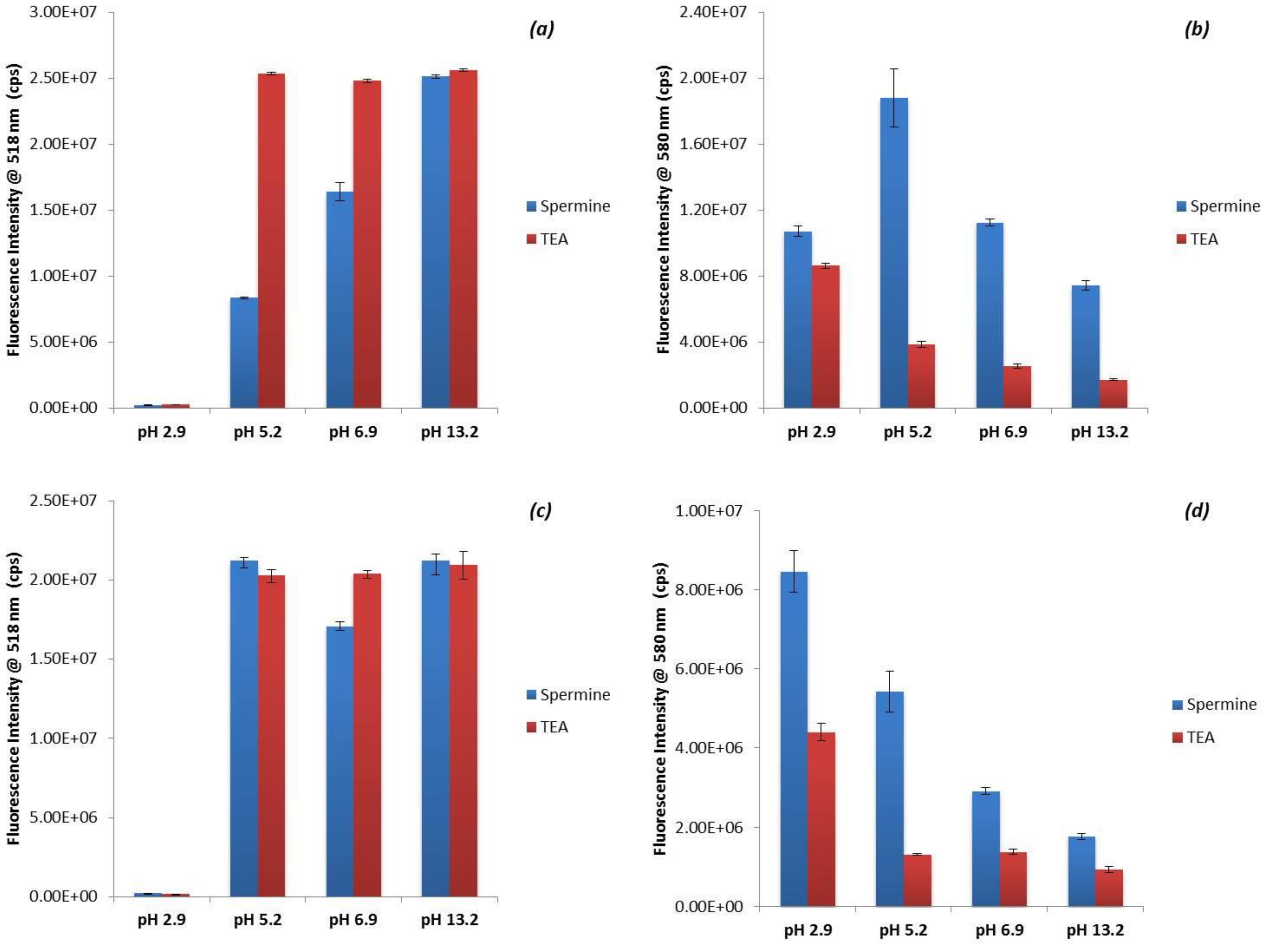

Figure 8 (a) Fluorescence intensity comparison obtained when TEA or spermine was added to a solution of FAM labelled single stranded DNA. (b) Fluorescence intensity comparison obtained when TEA or spermine was added to a solution of TAMRA labelled single stranded DNA. (c) Fluorescence intensity comparison obtained when TEA or spermine was added to a solution of FAM labelled double stranded DNA (d) Fluorescence intensity comparison obtained when TEA or spermine was added to a solution of TAMRA labelled double stranded DNA. For each experiment, 3 replicate samples were analysed 5 times and the error bars represent ± 1 standard deviation.

The major reason for this study was to establish the extent to which the emission from fluorescent dye labelled oligonucleotides would be affected under different conditions thus providing the knowledge to construct assays for more than one target. The two dye labelled targets were studied separately here as more complexity is to be expected if they are mixed. However, the data shown is somewhat surprising. In

fluorescence hybridisation assays, the relative intensities of the fluorescence from the two fluorescent dyes vary quite significantly with pH and concentration and evidence of DNA aggregation causing reduced fluorescence was also observed. In addition, the formation of a double strand alters the emission efficiency significantly. If a base is added, quite large changes in emission efficiency can occur and the magnitude is dependent on the base used. Spermine, used in SERS and enhanced fluorescence assays,[9, 10] has a significant effect on FAM fluorescence but only a small effect on TAMRA whereas TEA causes a large pH sensitive reduction in fluorescence in TAMRA and at some pHs a large enhancement of FAM fluorescence. For a deeper understanding as to what is happening on a molecular level, further computational analysis would need to be performed. However, the results reported herein highlight the significant consideration required when choosing the experimental conditions and reagents when fluorescence assays using multiple fluorophores are developed. Indeed, even for single fluorescent dye label assays, the addition of a base at the correct concentration and pH could greatly increase the efficiency of emission.

Conclusions

Investigative studies have shown that fluorescent dyes which are widely used in biological detection assays are susceptible to interactions with the DNA sequence they are covalently attached to and to the polyamine spermine. Both interactions have a significant effect on absolute fluorescence intensity which can potentially impact the use of these fluorescent labels in detection assays. The polyamine spermine is widely used in surface enhanced based detection methods; therefore it was of great importance to consider the interaction of this polyamine with fluorescent dyes as the overall signal obtained can be affected. Two fluorescent dyes were used throughout the investigation, FAM and TAMRA. When spermine was added to FAM labelled oligonucleotides, there was significant fluorescence enhancement. This was a result of the reduction in quenching by the DNA bases experienced by FAM as the spermine would intercalate between adjacent strands and reduce the quenching effect. Conversely, TAMRA did not exhibit the same fluorescence enhancement as observed in FAM. TAMRA is a positive fluorescent dye and therefore the polycation spermine would not have a strong interaction with this dye, compared to the negatively charged FAM dye. The fluorescence intensity is dependent upon experimental conditions such as spermine or oligonucleotide concentration and pH; however the fluorescence enhancement resulting from the addition of spermine is independent of the concentration of spermine and pH. The results from this study are of major consideration to the development of multiplexed DNA detection assays utilising spermine as it can enhance the fluorescence of some fluorescent dyes such as FAM and if this was in the presence of TAMRA, which does not experience fluorescent enhancement, will impact the signals obtained.

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Using fluorescence spectroscopy, spermine is shown to affect the emission of FAM and TAMRA, impacting the design of SERS-based assays.