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Development of a novel fluorescent tag O-2-[aminoethyl]fluorescein for the electrophoretic separation of oligosaccharides

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ABSTRACT

This study describes the development of a novel fluorescent tag, O-2-[aminoethyl]fluorescein, for the separation of sugars by capillary electrophoresis with fluorescence detection using an argon ion laser. The tag was synthesised using three consecutive steps namely: esterification, alkylation and hydrolysis, specifically designed to offer a flexible way in which to make an assortment of fluorescent tags from cheap and readily available starting reagents (typically less than $1 per g of fluorescent tag). Via this flexible synthetic pathway, O-2-[aminoethyl]fluorescein was designed and synthesised with a spacer group to lower steric effects between the fluorescein backbone and the reducing end of the carbohydrate which were anticipated to improve the reactivity of the tag. The newly synthesised tag, O-2-[aminoethyl]fluorescein was evaluated against structurally similar commercial fluorescent motifs namely fluorescent 5-aminomethylfluorescein and non-fluorescent 5-aminofluorescein. Kinetic studies indicated that O-2-[aminoethyl]fluorescein showed similar labeling efficiencies as 5-aminomethylfluorescein, but were achieved in only 30 min, supporting the notion of improved reactivity of the spacer group. The sensitivity of O-2-[aminoethyl]fluorescein was evaluated using maltoheptaose with a detection limit of 1 nM obtained, which was slightly higher than that of 0.3 nM obtained with 5-aminomethylfluorescein, and was due to its lower quantum yield (0.24) when conjugated to the sugar. The separation performance of the tag was also benchmarked with the two commercial reagents using a range of corn syrup oligosaccharides, from 4 to 10 glucose units, typically found in rice starch. Separations were performed using an electrolyte containing 100 mM boric acid, tris at pH 8.65 as background electrolyte, 30 kV applied voltage, 50 μm I.D. × 40 cm (30 cm effective length) capillary. The novel tag showed better resolution of small oligosaccharides, G3 and G4, than the other two reagents, but slightly worse resolution for the longer oligosaccharides, most likely due to the monovalent charge state of the O-2-[aminoethyl]fluorescein compared to the divalent charge of the other two tags.

1. Introduction

Today there is a growing demand for the analysis of various biomolecules such as carbohydrates, oligosaccharides and glycoproteins. These groups of compounds are the most numerous among the biopolymers and are found to play pivotal roles in biological processes such as tumour malignancy, viral infections and antigen/antibody biochemistry [1]. Further applications range from the food industry which encompasses microbiological studies, to the global issues of the carbohydrates as organic carbon deposits, directly affecting the carbon cycle [2–5].

A variety of analytical methods can be used to analyse these biopolymers such as high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), capillary gas chromatography (CGC) and mass spectrometry (MS) [6]. All of these methods have some limitations: HPLC shows difficulties resolving structural isomers, SFC and CGC require labeling of chain carbohydrates to improve solubility and volatility respectively, while MS requires internal standards to obtain necessary quantitation. Size exclusion chromatography (SEC) is another useful method to study carbohydrates although the technique is unable to separate smaller sugars and therefore requires additional methods to analyse a complete range of these biopolymers. Other techniques such as nuclear magnetic resonance (NMR) have been trialled however this lacks sensitivity and is inappropriate when dealing with small sample quantities [7]. CE is excellent for the separation of carbohydrates as it is complementary to SEC and it offers better separation efficiency, shorter separation times and smaller sample volumes [8] than other HPLC techniques. Even though CE is a preferred method for this analysis, the separations can be demanding as most

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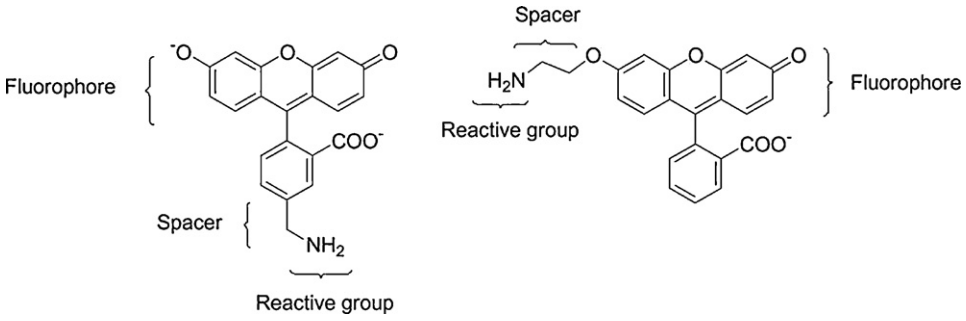

Fig. 1. Fundamental design of the molecule consisting of three main elements: a fluorescent moiety, a spacer and a reactive group.

carbohydrates are uncharged and do not contain any chromophore or fluorophore. Therefore derivatisation is usually necessary with a highly absorbing or fluorescing reagent. The use of LIF is quite attractive as it provides extremely low detection limits allowing better insight into carbohydrate analysis. Derivatisation reagents must not only have good fluorescence properties, but they must also contain a charged group to allow the carbohydrates to be separated by electrophoresis.

A variety of fluorophores for the separation of carbohydrates by electrophoresis have been reported in the literature and with the older literature summarised in reviews by Lamari et al., Paulus et al., Shilova et al., and Gao et al. [9–12]. These reviews cover a broad spectrum of various tags showing several detection methods and different derivatisation strategies to label carbohydrates, glycoconjugates and related compounds.

Careful evaluation of the research involving applications of fluorescent labels, tends to concur that 8-aminopyrene-1,3,6-trisulfonic acid (APTS), currently is the state of the art, commercially available label, which has been frequently used over the years, offering limits of detection in the order of 10^{-12} M [13]. While being the best existing fluorescent tag, APTS still has various limitations including its extremely high cost and relatively low molar absorptivity which can potentially diminish detection sensitivity. As opposed to APTS, fluorescein is a useful fluorescent molecule due to numerous reasons and a fluorescein-based carbohydrate reagent would have some appeal. Its quantum yield is significantly higher than for APTS, implying enhanced fluorescence, followed by better suitability for the argon laser and a high molar absorptivity in the range of 80,000 L mol^{-1} cm^{-1} [14]. The fluorescent tag also has charge bearing carboxylic acid and phenol functions, introducing mobility for electrophoretic studies. However some modifications are required to introduce the amino function necessary for labeling. Recent studies by Du et al. showed that a fluorescein backbone is gaining growing popularity for HPLC applications where they synthesised a 6-oxy-(acetyl ethylenediamine)fluorescein derivative and successfully labelled 12 fatty acids. Deng et al. also prepared a fluorescein derivative, namely *N*-hydroxy-succinimidyl fluorescein-*O*-acetate and applied it to quantitate biogenic amines with impressive 0.2–0.4 nM limits of detection [15,16].

This current study proposes a method for synthesising fluorescent tags based on the commercially available, yet expensive, aminomethylfluorescein as a model. The fundamental design of the molecule considers three main elements: a fluorescent moiety, a spacer and a reactive group (Fig. 1) where the latter group is an amine that can undergo reductive amination to label carbohydrates. Typically fluorescein derivatives have the functional groups attached at the benzene moiety, which allows dianion formation **II**, however an alternative approach is investigated to incorporate the spacer and the reactive group assembly onto the phenolic site of the fluorescein as shown in **I**. Such attachment modified the charge bearing phenolic function, resulting in formation of a monoanion, therefore reducing the mobility of the tag, but still making it suitable for CE analysis.

Ideally the synthetic approach adopted would be flexible where a specific intermediate substrate could be modified to construct an assortment of fluorescent analogues, with varied spacer groups. The advantage of the spacer assembly is evident since steric effects are minimised, resulting in a more nucleophilic aliphatic amine, which should provide better labeling efficiency and reactivity. Also by separating the amine from the fluorophore there is no fluorescence quenching due to the reduced donor effects from the amine group [17].

This study also evaluates the ability of the newly synthesised tag to label and separate a range of oligosaccharides obtained from corn syrup. To ensure that a meaningful assessment of the tag is made other fluorescein-based motifs are incorporated and compared throughout the study.

2. Experimental

2.1. General procedures and materials

Commercially available sources were used to purchase reagents and materials. There was no further purification of the reagents unless stated otherwise in the manuscript.

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectroscopy were recorded on a Varian Mercury 2000 Spectrometer operating at a frequency of 300 MHz and 75 MHz respectively.

Chemical shifts were recorded as δ values in parts per million (ppm) with a reference to the solvent applied. The solvents used were deuterated chloroform ($CDCl_3$), and dimethyl sulfoxide (DMSO-d_6). The following abbreviations are used to assign ^{1}H NMR spectra; s = singlet; d = doublet; t = triplet; bd = broad doublet; *J* = coupling constant (Hertz).

A Perkin Elmer FT-IR spectrophotometer Paragon 1000, was used to record infrared spectra. Solids and liquids were recorded as thin films on sodium chloride plates unless stated otherwise.

A Finnigan LCQ ion trap mass spectrometer with electrospray ionisation source was used to determine molecular weights of compounds. The samples were diluted in water and nebulised at 20 μL^{-1} min with a needle voltage of 4.9 kV, and a capillary temperature of 21 °C. The experiments were conducted under an inert atmosphere of N_2 gas.

Fluorescence spectra were recorded using a Perkin-Elmer 650-10S Fluorescence Spectrophotometer where the scanning rate of emission wavelength was set to 300 nm min^{-1}.

A Shimadzu UV–Visible Recording Spectrophotometer UV-160 was utilised for recording absorption spectra. The samples were diluted in water and set to scan the spectrum range between 400 nm and 800 nm.