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Development of near-infrared fluorophoric labels for the determination of fatty acids separated by capillary electrophoresis with diode laser induced fluorescence detection†

The Analyst

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Received 18th May 1999, Accepted 17th August 1999

Published on 01 January 1999. Downloaded on 15/01/2016 11:16:48.

Synthesis and characterization of a polymethine cyanine near-infrared (NIR) fluorophoric label for the derivatization and determination of fatty acids separated by capillary electrophoresis are described. The label contains an aromatic amine functionality, which is used to form a covalent linkage with the analyte. Various linking chemistries are explored, including direct amine–acid condensation using dicyclohexylcarbodiimide (DCC) as a carboxyl activating group. Spectrofluorimetry was used to probe the fluorescence efficiency of the label in order to assist in choosing a separation medium for capillary electrophoretic separation. A nonaqueous separation medium for capillary zone electrophoresis was used to provide high quantum efficiency for fluorescence and adequate solubility of fatty acid analytes. Diode laser-induced fluorescence detection following electrophoresis of a simple mixture of labeled fatty acids shows the applicability of this method to biologically relevant carboxylic acid analytes.

Introduction

Laser-induced fluorescence (LIF) has been shown to be one of the most sensitive methods available for detection in capillary electrophoresis.[1] LIF in the near-infrared (NIR) region of the spectrum is an attractive alternative to visible fluorescence for use in biological assays for several reasons. These include, but are not limited to, reduced fluorescence background, due to the relative absence of native fluorescent impurities in the near-infrared region, and diminished Raman scattering from solvent. In addition, NIR-LIF instrumentation is readily available, compact, rugged, and highly sensitive. Inexpensive semiconductor diode lasers can be used as high-powered sources. The use of silicon avalanche photodiodes (APD's) which have a detection efficiency of greater than 60% in the NIR region, allows for highly sensitive detection of fluorescence photons.

Near-infrared fluorescence has already been demonstrated to be a viable alternative to visible fluorescence for a number of bioanalytical assays. To date, the majority of NIR dyes modified for derivatization have used active groups designed for coupling with amino groups of amino acids and proteins.[2–4] Traditionally, dyes of this type have been synthesized in a condensation reaction between two equivalents of a quaternary salt of a heterocyclic base containing an activated methyl group with an unsaturated bisaldehyde.[5] The resultant symmetrical dye contains a labile chloride moiety in the polymethine backbone which can be substituted with a number of reactive functional groups designed for covalent labeling of a variety of analytes. Substitutions on the heterocyclic base can be made to tailor the solubility and spectral properties of the dye. General characteristics of these polymethine cyanine dyes include large molar absorptivities ($>100{,}000$ cm^{-1} M^{-1}) and spectral properties (absorption and emission maxima) in the range of 700–1100 nm.

It has been estimated that approximately 8% of organic compounds of pharmaceutical and/or biomedical interest possess a carboxylic acid functional group.[6] Therefore, there is much interest in developing sensitive assays for compounds such as free fatty acids, bile acids, eicosanoids, and various pharmaceuticals such as the non-steroidal anti-inflammatory drugs (NSAIDS). Direct assay for these compounds at trace levels can be difficult, especially in the case of fatty acids, due to the lack of native fluorescence and the low UV absorptivity of the carbonyl group ($\log \varepsilon < 2$). Fatty acids are of particular interest for several reasons. For example, fatty acid profiles of human sera have been shown to be markers for diseases such as diabetes and thyroid dysfunction.[7] A review by Gutnikov discusses methodologies for characterizing lipid samples by their fatty acid profile.[8] Previous analyses of fatty acid analytes by capillary electrophoresis have focused on micellar electrokinetic chromatography (MEKC) or isotachophoresis with indirect UV absorbance or electrochemical detection.[9,10] To our knowledge, this work represents the first time that NIR fluorescent derivatization has been applied to the determination of fatty acids.

We report here the development of a polymethine cyanine near-infrared fluorophoric label designed for coupling with the carboxy group of fatty acids. The label has been previously reported as a precursor to isothiocyanate-based labels for primary amines.[5] The label possesses an aromatic amine moiety covalently attached to a polymethine cyanine skeleton. The amino group is used to form a covalent linkage to the fatty acid analyte by either conversion of the acid to an acid chloride, followed by derivatization; or direct amine–acid condensation using dicyclohexylcarbodiimide (DCC), a reagent commonly used in peptide synthesis. The label is synthesized to possess an absorption maximum corresponding to the output of a common InGaAlAs single mode laser diode (780 nm). The solubility of the label is tailored to non-polar organic solvents for both derivatization concerns and for the highest quantum efficiency.

† Presented at FACSS XXV, Austin, TX, USA, October 11–16, 1998.

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Experimental

Instrumentation

Absorption spectra were recorded on a Varian Cary model 3E spectrophotometer. Spectrofluorimetry was performed on a Photon Technologies International QM-1 system equipped with a red-sensitive photomultiplier tube. Infrared spectra were recorded on a Perkin Elmer 1600 series FT-IR. Nuclear magnetic resonance spectra were recorded on a Brüker ACP-300 Multinuclear FT-NMR Spectrometer. Capillary electrophoresis separations were performed using an instrument constructed in-house, the details of which are described below.

Materials and reagents

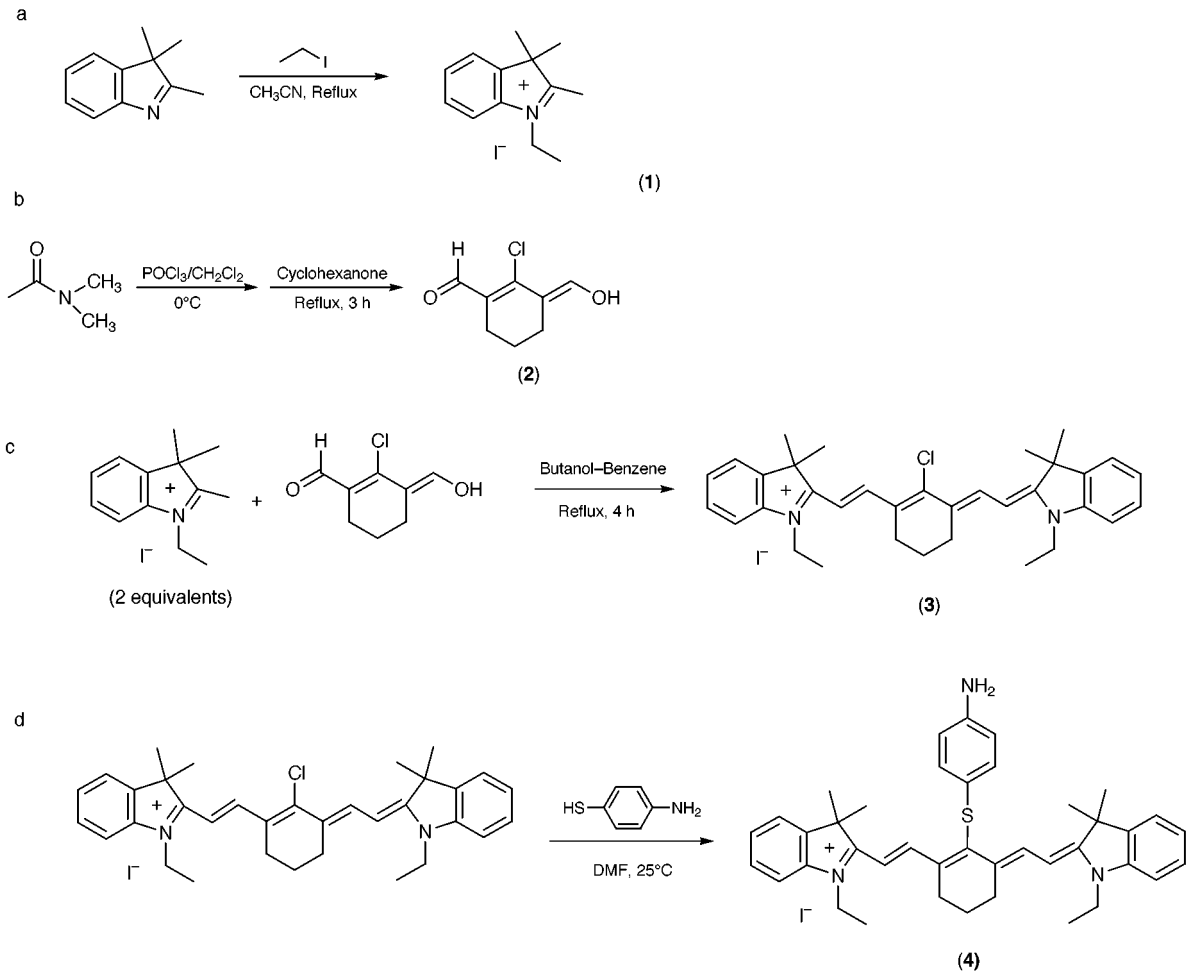
Reagents for the dye synthesis and derivatization schemes were of the highest purity available and obtained chiefly from Acros Organics (Fisher Scientific, Pittsburgh, PA). Fatty acids palmitic (C16), lauric (C12), oleic (C18:1), caproic (C6), and propionic were from house stores originally purchased from Fisher Scientific (Fairlawn, NJ). Valeric (C5) and capric (C10) acids were from house stores originally purchased from Eastman Organics (Rochester, NY). 4-Aminothiophenol was purchased from Aldrich Chemical (Milwaukee, WI). Water for synthesis and solution preparation was purified using a Barnstead Nanopure system. Organic solvents from house stores were of reagent grade or better and were further purified for use by distillation using all-glass stills and were dried over molecular sieves before use.

Synthetic methods

The NIR fluorophoric label was synthesized using a modified procedure from that of Narayanan, *et al.*[5] An outline of the synthesis pathway is given in Scheme 1.

(i) Synthesis of *N*-ethyl-2,3,3-trimethylindolinium iodide (1). The heterocyclic base containing an activated methyl group was synthesized as follows: 2,3,3-trimethylindolinene (0.025 mol, 99%) and iodoethane (0.125 mol) were combined in 100 mL dry acetonitrile and the mixture was purged with nitrogen to remove dissolved oxygen. The mixture was then refluxed for 24 h under a light blanket of N_2. After the reaction period, the solvent was removed *in vacuo* and the residue was purified by crystallization in diethyl ether. Solvent removal *in vacuo* yields 7.74 g, 0.0245 mol (99%).

(ii) Synthesis of 2-chloro-1-formyl-3 hydroxymethylene cyclohexene (2). 40 mL of dry dimethylformamide (DMF) was dissolved in 40 mL of methylene chloride and cooled in an ice bath to 0 °C. In a separate vessel, 37 mL of phosphorus oxychloride was dissolved in 35 mL of methylene chloride, and this solution was added to the solution of DMF dropwise with continuous stirring at 0 °C. After the addition was complete, 10 g of cyclohexanone dissolved in 25 mL of methylene chloride was added, dropwise with stirring. The solution was brought to room temperature and then refluxed for three hours. Upon cooling, the solution was cautiously poured onto 200 g of ice and allowed to warm to room temperature. It was discovered that if the solution was allowed to sit overnight, according to the literature procedure, the yellow bisaldehyde became contaminated with reaction by-products present in the biphasic system.



Scheme 1 Synthesis pathway for the near-infrared fluorophoric label.

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The material isolated from this method proved impure despite repeated recrystallizations. Thus, the solution was poured immediately into a separatory funnel, the lower layer was drawn off and the yellow bisaldehyde was immediately isolated from the aqueous solution by vacuum filtration and washed with cold water. The yellow needles were dried *in vacuo*, yield 8.2 g, 0.047 mol (47%).

(iii) Synthesis of symmetric chloro dye I (3). 2.60 g (0.008 mol) of *N*-ethyl-2,3,3-trimethylindolinium iodide were combined with 0.77 g (0.004 mol) of 2-chloro-1-formyl-3 hydroxymethylene cyclohexene in 150 mL of dried 7:3 *n*-butanol:benzene in a 250 mL round bottom flask. The reaction mixture was purged with N_2 to remove dissolved oxygen and the mixture was refluxed for four hours in a reflux apparatus equipped with a calcium sulfate drying tube. At the end of the reaction period, the flask was cooled and the solvent was removed *in vacuo*. The residue was crystallized in diethyl ether and purified by column chromatography on a silica gel column using a methanol–chloroform gradient. Fractions having an absorbance maximum of 777 nm (methanol) were combined and the solvent was removed *in vacuo* to yield 2.2 g (0.0035 mol, 79%) purified dye. The dye was stored at 0 °C under Ar in the dark.

(iv) Functionalization of symmetric chloro dye I to fatty acid label (4). To a 100 mL round bottom flask was added 0.063 g (0.1 mmol) of purified dye and a magnetic stir bar. The flask was capped with a rubber septum and the vessel was purged with dry N_2. 5 mL of anhydrous DMF was added *via* a syringe, with stirring to dissolve the dye. 0.070 g (0.55 mmol) 4-aminothiophenol was dissolved in one milliliter of anhydrous DMF and injected through the septum. The mixture was stirred for five minutes at ambient temperature, and the DMF was removed *in vacuo*, keeping the temperature of the water bath below 50 °C to prevent decomposition of the label. The residue was purified by column chromatography using identical conditions as in the previous purification. Solvent removal *in vacuo* yields 0.059 g (0.082 mmol, 82%). ^{1}H NMR (300 MHz, methyl sulfoxide-d_6) shows insertion of the aromatic amine into the skeleton by a broad peak at 5.6 ppm (NH_2) and doublet peaks of the aromatic ring at 7.4 and 7.6 ppm. The purified label was stored at 0 °C, under Ar, in the dark.

Derivatization protocol

Stock solutions of the NIR label were prepared in methyl sulfoxide. Stock solutions of various fatty acids and DCC were prepared in chloroform . A typical derivatization procedure was as follows: 100 μL of a 1 M stock solution of fatty acid was added to a conical glass reaction vial with a magnetic stir bar. 120 μL of a 1 M stock solution of DCC was then added and the mixture was stirred for 30 s. An aliquot of the NIR derivatization reagent in methyl sulfoxide (at a concentration to achieve an equimolar ratio of label to the fatty acid) was added, and the vial was capped and allowed to stir in the dark for 24 h. At the end of the reaction period, precipitated dicyclohexylurea (DCU) was filtered off with a 0.2 μm membrane filter and the mixture was diluted with methanol. Analytical samples for CE analysis were diluted in the CE separation medium. For the analysis of mixtures, aliquots of individual reaction products were pooled and serially diluted with CE separation medium.

CE-LIF detection system

The LIF detection system was constructed as follows: excitation at 780 nm was provided by a InGaAlAs single mode laser diode (Sharp LTO27MD), collimated using an aspheric collimating lens (Thorlabs, Inc., Newton, NJ) and circularized using an external anamorphic prism pair (Melles Griot, Irvine, CA). The beam was focused into the capillary using a 40.0 mm achromat doublet lens (Melles Griot). Fluorescence was collected at 90° with a 40X, 0.85 NA microscope objective (Fluor 40, Nikon, Tokyo, Japan). The objective focused the fluorescence onto a 400 μm pinhole (Melles Griot) placed in the primary image plane of the objective. The fluorescence was filtered immediately after the pinhole by an interference bandpass filter at 850nm±20nm (Omega Optical, Brattleboro, VT). The remaining fluorescence was collected and focused by a pair of achromat lenses (12.5 mm diameter, 24 mm EFL, Rolyn Optics, Covina, CA), onto the active area of an actively quenched, photon counting, avalanche photodiode (SPCM-AQ-131, EG&G Optoelectronics Canada, Vaudreuil, Quebec). The SPCM also amplified and discriminated the photoelectron pulses, and the TTL output was sent to a multichannel scaler card (MCS II, Oxford Tennelec/Nucleus, Oak Ridge, TN) residing in a Dell Optiplex 466/MXe. Data collection was controlled by software supplied with the MCS hardware; data was further analyzed using Igor Pro (Wavemetrics, Lake Oswego, OR). Because of the 90° excitation/emission format, alignment of the emission train was accomplished by back-reflecting the beam of a HeNe alignment laser (Melles Griot) through the train to assure orthogonality to the excitation train.

Capillary electrophoresis

Separations were performed in a 50 μm internal diameter, 79 cm (45 cm injection to detection) bare fused silica capillary (Polymicro Technologies, Phoenix, AZ). Detection windows were prepared by removing a section of the polyimide coating with a low temperature flame. New capillaries were treated by flushing with distilled, deionized water for 30 min, followed by 1.0 M NaOH for 30 min, followed by distilled, deionized water for 30 min. The capillary was then filled with separation medium and allowed to stabilize under high voltage. The medium for all separations was methanol with 12.5 mM tetraethylammonium chloride. High voltage power was supplied by a CZE 1000R (Spellman High Voltage Electronics Corporation, Plainview, NY). Injections were performed electrokinetically (3 s, 10 kV), and separations were run at 25 kV (7 μA). For all separations, the detection end of the capillary was held at ground potential and high voltage was applied to the injection end. All separations were carried out at ambient temperature.

Results and discussion

Derivatization of fatty acids

In the recent literature there have been a number of reports of fluorophoric reagents designed for covalent labeling of fatty acids. Many are based on the alkylation of carboxylate anions. Reagents such as 4-bromomethyl-7-methoxycoumarin (Br-MMC) react with carboxylic acids in the presence of phase-transfer catalysts such as crown ethers in polar, aprotic solvents.[11] A variety of coumarin analogs have been prepared and used to label a range of carboxylic acids. Zuriguel *et al.* have reported the separation of C8 to C11 fatty acids derivatized with 5-bromomethylfluorescein in which they achieve a sub-nanomolar concentration limit of detection.[9] Other labeling chemistries have been developed, which include reaction of carboxylic acid analytes with fluorescent hydrazides, diazoalkanes, alcohols, anthracenes, and aromatic amines. An extensive review of derivatization reagents for carboxylic acids has been published by Mukherjee and Karnes.[6]

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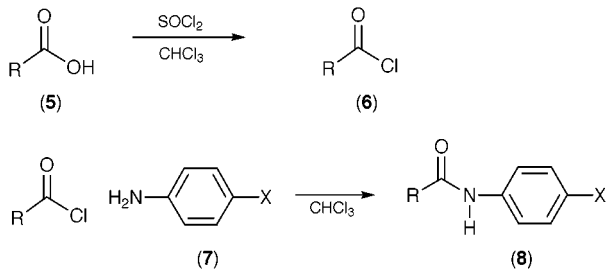
Reaction of fatty acid analytes with an amine-based fluorophoric label is attractive in the scope of this work due to the ease of modifying the heptamethine cyanine fluorophores to incorporate an aromatic amine in the polymethine backbone. The labile chlorine moiety in the skeleton of the unfunctionalized dye (**3**, Scheme 1) can be replaced in a substitution reaction with 4-aminothiophenol or 4-aminophenol. The disadvantage to using 4-aminophenol to functionalize the dye is the lack of reactivity of the hydroxy group in displacing the chlorine. The hydroxy group is usually deprotonated with a hydride base, which requires initial protection of the amine with di-*tert* butyldicarbonate or a similar protecting agent, increasing the complexity of the synthesis and reducing the overall yield. In contrast, reaction of 4-aminothiophenol with symmetric chloroheptamethine cyanine dyes readily produces the amine functionalized fluorophore (**4**) under mild conditions at room temperature.

Amines can be covalently coupled to carboxylic acids in a variety of ways. One common method is illustrated in Scheme 2. This derivatization pathway involves initial conversion of the carboxylic acid (**5**) to an acid chloride (**6**) with thionyl or oxalyl chloride followed by reaction with an aromatic amine (**7**). The resultant product, a secondary amide, has the advantage of being stable towards hydrolysis which is attractive from the standpoint of derivatization, separation, and detection. Hoffman and Liao have used this method of derivatization to incorporate a UV chromophore (1-naphthylamine) into fatty acids for absorbance detection following separation by HPLC.[12] Attempts to derivatize free fatty acids with an amine-based NIR fluorophore using this pathway proved unsuccessful. Electropherograms of reaction mixtures showed only one peak, corresponding to the labeling reagent. NMR spectra taken of column chromatography fractions of reaction mixtures revealed cleavage of the amino thiol linker arm from the backbone of the dye skeleton. It was thought that HCl liberated from the derivatization caused the aminothiol linker arm cleavage, but successive experiments with varying concentrations of pyridine or triethylamine as HCl scavengers produced no derivatized product.



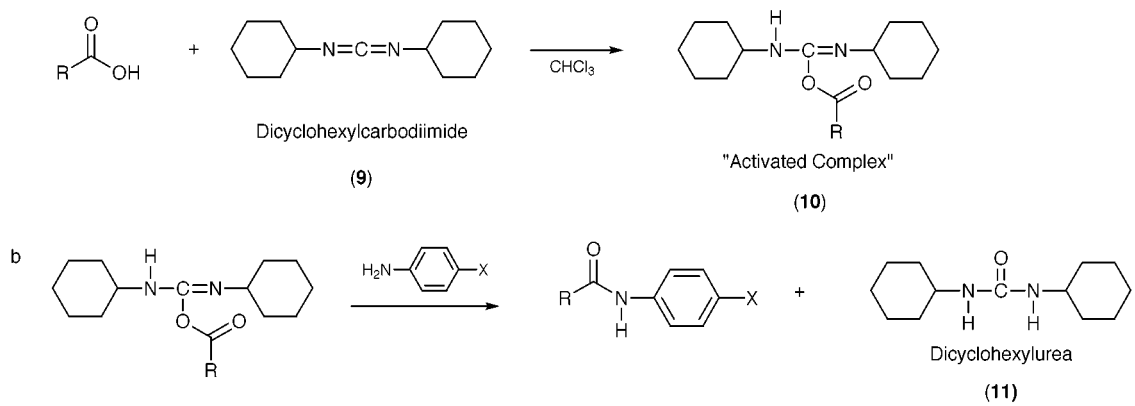
(**5**) (**6**) (**7**) (**8**)

Scheme 2 Pathway for derivatization of a carboxylic acid with an aromatic amine *via* initial conversion to an acid chloride.

Another method which has been demonstrated for covalently labeling carboxylic acid analytes with amines is the direct acid–amine condensation using dicyclohexylcarbodiimide (DCC). This derivatization pathway is illustrated in Scheme 3. DCC (**9**) functions by activating the carbonyl group of the carboxylic acid (**10**) towards nucleophilic attack by the amine to form the amide and highly insoluble dicyclohexylurea (**11**). DCC has been used extensively in peptide synthesis as a fast and efficient coupling reagent.[13] Fluorescent carbodiimides have been used in the absence of nucleophiles to form fluorescent derivatives of carboxylic acid residues on proteins, but the majority of reports using DCC to derivatize carboxylic acid analytes have incorporated a UV chromophore for absorbance detection.[6] DCC-promoted condensation has proved to be an efficient method for coupling free fatty acids with the amine-based NIR label.

Kinetics and reaction efficiency

Studies of reaction rate at concentrations of 0.3 M fatty acid and 20-fold excess of fatty acid to fluorescent label yielded a reaction half-life of less than five minutes for C5 and C10 fatty acids. Half-lives for ten-fold dilute reactions in fatty acid were approximately one order of magnitude greater. These half-lives are for the overall reaction; as shown in Scheme 3, two reactions with multiple reagents occured sequentially in a single 'pot', and the term 'half-life' can only be interpreted loosely without detailed kinetic data. At 0.03 M fatty acid concentrations, the induction effect for appearance of product was apparent. These reaction rates were not fast enough for on-column derivatization, but were adequate for typical, pre-column conditions (*i.e.* overnight incubation). Acids up to C22 have been consistently derivatized at concentrations as low as 10^{-4} M. Preliminary results suggested successful derivatization at concentrations at least as low as 10^{-7} M in overnight reactions, which was roughly consistent with known performance of DCC couplings in peptide synthesis, in which micromolar concentrations are commonplace. This reaction therefore has the potential to allow quantitative derivatization at useful concentrations (sub-micromolar). Based on relative peak heights, reaction efficiencies were greater than 98% at high concentrations (0.3 M), and dropped to 90% at ten-fold lower concentrations. Longer chain acids exhibited slightly lower efficiencies (*e.g.* 84% for C18). Fortunately, this chemistry produced no labeling reaction byproducts, and the reaction product (a secondary amide) was quite stable, so elevated temperatures and extended reaction times could be used to increase the yield of the derivatization reaction. Products have been shown to be stable for at least two weeks. In addition, the formation of the amide bond was irreversible; in other words, the reaction was not an equilibrium, and lower concentration derivatizations were limited only by kinetics. However, temperatures in excess of 50 °C must be



Scheme 3 Pathway for derivatization of a fatty acid by direct acid-amine condensation promoted by dicyclohexylcarbodiimide (DCC).

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avoided in order to prevent decomposition of the labeling reagent.

Basic spectroscopy and separation

UV-vis-NIR spectroscopy and spectrofluorimetry were used to qualitatively probe the unfunctionalized dye and the amine functionalized label to aid in the choice of separation medium. Table 1 shows absorption and emission maxima for the unfunctionalized dye and the functionalized label. There was a noticeable red shift in the absorbance maxima of the functionalized label (**4**) from that of the unfunctionalized dye (**3**). This was most likely due to extended conjugation through the expanded π system upon addition of the aromatic amine moiety. The emission maxima of the functionalized label were slightly blue shifted from that of the unfunctionalized dye. The fluorescence behavior of polymethine cyanine dyes has been thoroughly investigated.[14,15] The relative quantum efficiencies of the functionalized label in various solvents can be seen in Fig. 1 and are collected in Table 1. The choice of an all-organic separation medium was guided by concerns for improved photophysics of the label[14,15] and increased solubility of the conjugated fatty acid in the medium. In addition, the use of an organic medium helped to minimize interactions between the cationic label and bare silica wall of the capillary, which can lead to a loss of resolution in the separation. The addition of 12.5 mM tetraethylammonium chloride as an electrolyte served as a charge carrier to match the conductivity of the separation medium with that of the sample zone.

The fatty acids selected for this work were chosen to cover a molecular weight range typical of biologically relevant fatty acids. Acids chosen included propionic (C3), valeric (C5), caproic (C6), capric (C10), lauric (C12), palmitic (C16), and oleic (C18:1). A typical electropherogram of a mixture of the labeled fatty acids is shown in Fig. 2. Peak identities were confirmed by the injection of individual standards and by spiking the mixture with aliquots of the individual standards. Labeled fatty acids eluted in order of increasing molecular weight. Adequate resolution was obtained for the majority of the acids examined, except for oleic which coeluted with lauric acid. This was presumably due to the unsaturation on the molecule. Simple simulations based on parameters from this separation suggested that all saturated fatty acids in this molecular weight range can be separated with a resolution similar to that achieved between C5 and C6 (R_s = 1).

More detailed studies of reaction efficiency and spectroscopic behavior are underway; however, it appeared that roughly the same amount of product was obtained in all reactions except that of propionic acid. Studies of DCC-promoted condensations of carboxylic acids have revealed that formation of the activated complex between DCC and an acid is kinetically favorable in a solvent in which the acid possesses limited solubility.[16] It was possible that the increased solubility of propionic acid, compared to the other acids, led to unfavorable reaction kinetics in the derivatization step.

Injected samples were diluted to give a concentration of 80 nM in fluorescent label. A calibration curve of functionalized label (**4**) showed excellent linearity (r^2 = 0.9999) over four orders of magnitude, with a monotonic rise in signal-to-noise ratio. The calculated concentration limit of detection (S/N = 3) was 60 pM in the methanolic separation medium, for undiluted (*i.e.* flat-top) peaks.

Table 1 Absorbance and emission maxima (nm) for the unfunctionalized dye (**3**) and functionalized label (**4**) in various solvents

	Dye		Label		
Solvent	Absorbance	Emission	Absorbance	Emission	Relative intensity
Cholorform	788	815	801	814	1.0
Methylene chloride	788	814	796	813	0.78
Ethyl acetate	782	809	789	808	0.63
Acetonitrile	777	806	782	804	0.62
Acetone	780	808	783	807	0.61
Methanol	777	806	787	805	0.42
Water	770	800	775	798	0.18

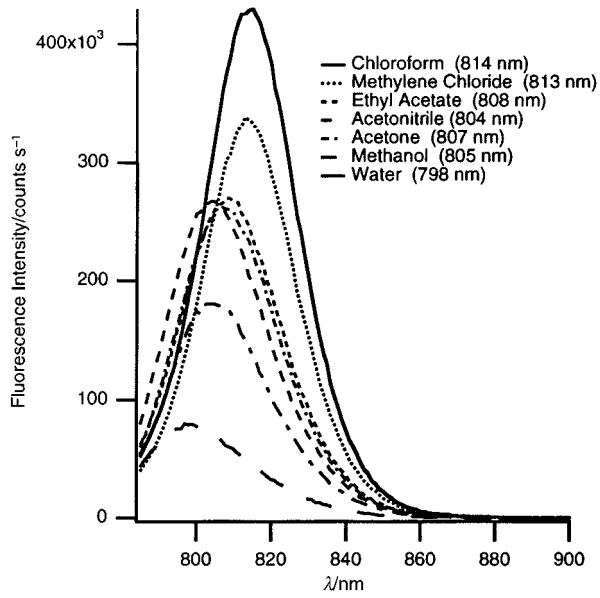

Fig. 1 Emission spectra of functionalized fatty acid label in various solvents. The concentration of the label was 4.5 μM, and the excitation wavelength was 780 nm. Positions of maxima are given in the legend.

Conclusions

We have demonstrated covalent labeling of linear saturated fatty acids with a polymethine cyanine near-infrared fluorophore. The kinetics and coupling efficiency were comparable to common visible dyes used for covalent labeling in LIF, but superior stability of the conjugates and excellent absorptivity, quantum efficiency (in nonaqueous solvents), and lower background at NIR wavelengths give this analysis scheme many advantages compared with other (visible) schemes. In addition, the fact that the use of nonaqueous solvents benefits both the separation (efficiency, solubility) and photophysics (sensitivity) of the label makes this a good combination for fatty acid analysis.

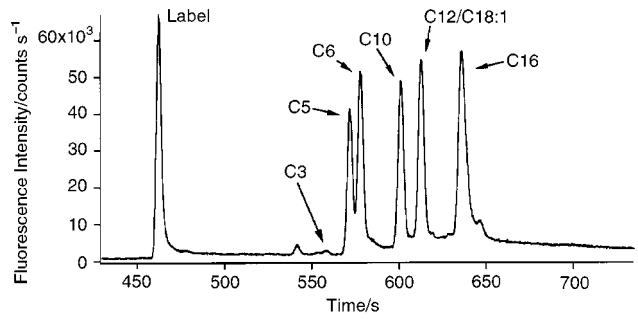

Fig. 2 CE-LIF separation of a mixture of fatty acids derivatized with the NIR label. See text for conditions.

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Acknowledgements

The authors thank the Duquesne University Faculty Development Fund for support of this work.

References

1 M. Albin, P. D. Grossman and S. E. Moring, *Anal. Chem.*, 1993, **65**, 489A.
2 D. B. Shealy, M. Lipowska, J. Lipowski, N. Narayanan, S. Sutter, L. Strekowski and G. Patonay, *Anal. Chem.*, 1995, **67**, 247.
3 M. Lipowska, G. Patonay and L. Stretkowski, *Synth. Commun.*, 1993, **23**, 3087.
4 A. J. G. Mank and E. S. Yeung, *J. Chromatogr.*, 1995, **708**, 309.
5 N. Narayanan and G. Patonay, *J. Org. Chem.*, 1995, **60**, 2391.
6 P. S. Mukherjee and H. T. Karnes, *Biomed. Chromatogr.*, 1996, **10**, 193.
7 M. Saito, T. Ushijima, K. Sasamoto, Y. Ohkura and K. Ueno, *J. Chromatogr. B*, 1995, **674**, 167.
8 G. Gutnikov, *J. Chromatogr. B*, 1995, **671**, 71.
9 V. Zuriguel, E. Causse, J. D. Bounery, G. Nouadje, N. Simeon, M. Nertz, R. Salvayre and F. Couderc, *J. Chromatogr. A*, 1997, **781**, 233.
10 F. B. Erim, X. Xu and J. C. Kraak, *J. Chromatogr. A*, 1995, **694**, 471.
11 W. Dünges, *Anal. Chem.*, 1977, **49**, 442.
12 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 1976, **48**, 1104.
13 M. Bodanszky, in *Peptide Chemistry: A Practical Textbook*, Springer-Verlag, Berlin, 1988, p. 55.
14 S. A. Soper and Q. L. Mattingly, *J. Am. Chem. Soc.*, 1994, **116**, 3744.
15 S. A. Soper, B. L. Legendre and J. Huang, *Chem. Phys. Lett.*, 1995, **237**, 339.
16 B. J. Balcom and N. O. Petersen, *J. Org. Chem.*, 1989, **54**, 1922.

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