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Long-Wavelength Absorbing and Fluorescent Chameleon Labels for Proteins, Peptides, and Amines

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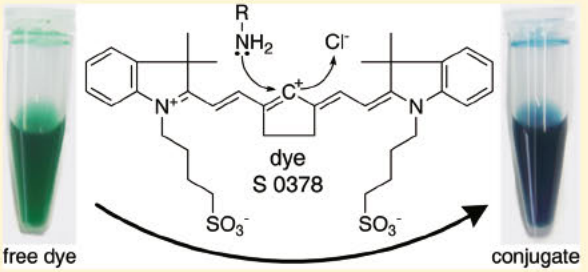
Supporting Information

ABSTRACT: Long wavelength absorbing labels that change their color and fluorescence upon conjugation to proteins and other biomolecules provide two critical advantages over the wealth of conventional amine reactive labels. At first, the progress of the labeling reaction can be monitored continuously either visually or by spectrometry without prior purification. Then, the labeled biomolecule can be investigated with red or near infrared light, which minimizes background interference in biological samples. These unique characteristics are met by a group of long wavelength absorbing cyanine dyes carrying a reactive chloro substituent for nucleophilic substitution with primary amines, which is accompanied by a color change from green to blue. In addition to this so called chameleon effect, the dyes display an increase in fluorescence during the labeling reaction. Despite their structural similarity, the reactivity of the dyes differs strongly. The fastest labeling kinetics is observed with dye S 0378 as its five membered ring affords a stabilizing effect on the intermediate carbocation during an S_N1 type of nucleophilic substitution. The reaction mechanism of the amine reactive cyanine dyes provides a blueprint for the design of future long wavelength absorbing chameleon dyes.



INTRODUCTION

Modification by amine reactive probes constitutes the prevalent route for labeling proteins, peptides, and other amines. These labels undergo an addition reaction (e.g., the isothiocyanates) or a nucleophilic substitution reaction (e.g., the sulfochlorides) with deprotonated amino groups of lysine and the amino terminus on the surface of proteins, or with amino groups of other biomolecules to form a stable covalent bond. The absorbance of most current labels is not affected by the labeling reaction, so any label that has not reacted cannot be distinguished spectrally from a label bound to a protein. Consequently, it is necessary to separate the free label from the labeled protein after the reaction has completed. To address this shortcoming of conventional labels, we have introduced pyrylium (Py) dyes as a group of so called chameleon labels that undergo a visually detectable color shift to a shorter wavelength as well as a change in fluorescence upon conjugation to amino groups.[1,2] As a result of this chameleon effect, the labeled protein can be easily differentiated even if the free label is present in large excess. Py labels have found various applications in areas such as protein assay and labeling,[3] in studies on neuropeptides,[4] gel electrophoresis,[5] capillary electrophoresis,[6−8] fluorescence based cytometric single cell analysis,[9] and in surface chemistry.[10] Py labels were also used in immobilized form.[11,12] Kostenko et al.[13] presented another class of chameleon labels which leads to long wave shifted rather than to shortwave shifted conjugates.

Long wavelength absorbing labels are of substantial interest because red and near infrared (NIR) light penetrates more deeply into biological materials and generates much less background fluorescence than short wavelength light.[14] Cyanine (Cy) dyes are a prominent class of fluorescent dyes that depending on their molecular structure cover the whole spectrum from the blue to the NIR.[15−17] Some red and NIR absorbing cyanine dyes are equipped with a chloro group in the center of their conjugated π electron system (Figure 1) that undergoes facile nucleophilic substitution by amines and thiols in organic solution at elevated temperatures.[18−20] Patonay et al.[18] substituted the chloro group by thiophenols followed by several modification steps to obtain amino reactive labels. This complex label design, however, omits a key feature of these cyanine dyes: If the electron withdrawing chloro group is substituted by an electron donating amine, the dye's absorbance strongly shifts to shorter wavelengths.[21] We perceived that this chameleon effect may be amenable to direct protein labeling in aqueous solutions and temperatures below 40 °C. To confer water solubility, we chose three exemplary cyanine dyes containing sulfo groups (Figure 1) and investigated them with respect to their reactivity toward proteins, peptides, and various amines.

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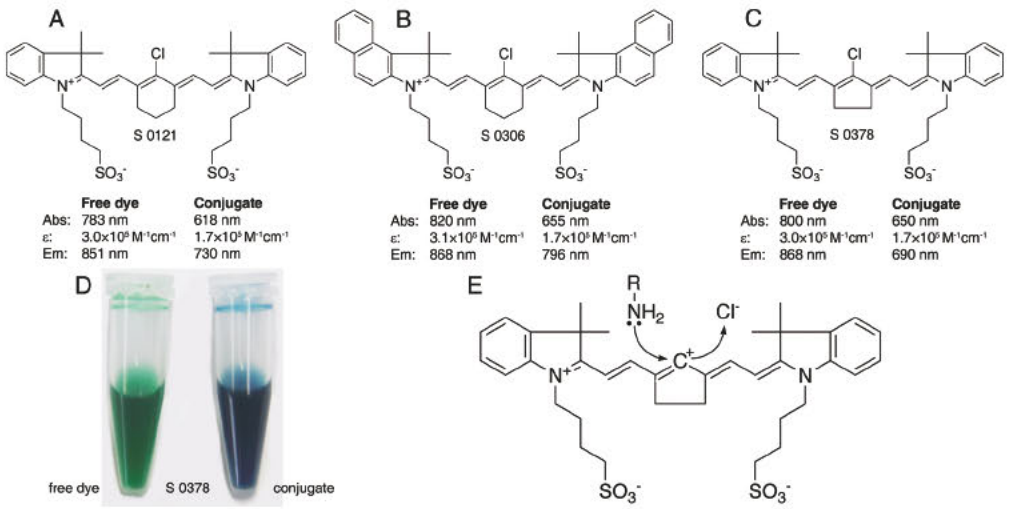

Figure 1. Chemical structures of the water soluble cyanine dyes S 0121 (A), S 0306 (B), and S 0378 (C). Conjugation to amines leads to a color change from green to blue and a change in the emission spectrum. Absorbance maxima, molar absorbances, and emission maxima of each dye and its conjugate to HSA are shown. (D) Photograph of microtubes containing dye S 0378 (200 μg/mL) in the unconjugated form (left) and conjugated to HSA (5 mg/mL, right). The labeling reaction can be followed visually by the color change from green to blue. (E) Nucleophilic substitution of dye S 0378 by primary amines. The five membered ring structure in combination with the polymethine chain sustains the formation of a carbocation and directs the reaction toward an S_N1 mechanism.

EXPERIMENTAL PROCEDURES

Materials and Instruments. Goat antimouse IgG antibodies and human serum albumin (HSA) were purchased from Sigma Aldrich (Munich, Germany; www.sigmaaldrich.com). The pep tide angiotensin II with the sequence H_2N Asp Arg Val Tyr Ile His Pro Phe COOH was purchased from Bachem (Weilam Rhein, Germany; www.bachem.com). The long wavelength ab sorbing cyanine dyes S 0121, S 0306, and S 0378 were obtained from FEW Chemicals (Wolfen, Germany; www.few.de). Other reagents and solvents were of the highest grade available. Size exclusion chromatography was performed using Sephadex G 75 (GE Healthcare, Munich, Germany; www.gehealthcare.com). Fluorescence spectra were acquired on an FP 6300 fluorescence spectrometer from Jasco (Essex, UK; www.jasco.co.uk) and mass spectra on a MALDI TOF mass spectrometer from GSG (Bruchsal, Germany; www.gsg analytical.com).

Labeling of Proteins and Peptides. Stock solutions of 10 mg of the dyes S 0121, S 0306, and S 0378 in 1 mL of methanol, as well as 1 mg/mL of HSA, IgG, and peptide in 20 mM sodium carbonate buffer, pH 8.4, were prepared. Two microliters of the dye solution and 200 μL of the protein/peptide solution were added to 20 mM sodium carbonate buffer, pH 8.4, to give a total reaction volume of 1 mL. The solutions were shaken in a thermomixer (950 rpm) at 37 °C for various periods of time. For further characterization, labeled proteins (blue) were pur ified from the free dye (green) by size exclusion chromatography on Sephadex G 75.

MALDI-TOF Mass Spectrometry. A small quantity of un labeled or labeled HSA was dissolved in 50 μL water and mixed in a 1:1 (v/v) ratio with an oversaturated solution of sinapic acid in methanol/acetonitrile (1:1, v/v). One drop of the mixture was deposited onto a metal plate and, after solvent evaporation, the spot was analyzed by MALDI TOF mass spectrometry.

Labeling of Primary Amines with Dye S 0378. Solutions of 272 μM of dye S 0378, 408 μM of primary amine (β alanine, dodecylamine, ethanolamine, histamine, propargylamine, or tyramine), and 408 μM of triethylamine in methanol were incubated either for 15 h at 25 °C or for 4 h at 70 °C.

RESULTS AND DISCUSSION

Selection of Long-Wavelength Absorbing Chameleon Dyes. Protein labeling is preferably performed in aqueous solutions and at ambient temperature in order to leave the protein structure intact. Furthermore, the labeling reaction is expected to be fast to save time and prevent microbial growth in a sample. On the other hand, the label has to be chemically stable with respect to storage and preventing spontaneous reactions in a sample.

The nucleophilic ε amino group of lysine presents a labeling site on proteins for which various amine reactive reagents are available. In most cases, the labels feature two distinct subsites: one for coupling to amines and the other one for generating a signal usually a conjugated π electron system that serves as a chromophore or fluorophore. As the coupling site and the conjugated π system are separated, the color or fluorescence of the label is not affected by the coupling reaction. Consequently, the labeling reaction is not detectable by a color change and the labeled protein needs to be purified from the free dye. In the chameleon dyes, by contrast, the π system takes part in the coupling reaction and the coupled amino group exerts an electron shifting effect. Polyenes, however, undergo addition reactions with nucleophiles, which would ultimately destroy the conjugated π system and prevents their use as a conjugation site. Aromatic rings that are part of the delocalized electron system may undergo substitution reactions by amines under conditions that are suitable for labeling proteins,[22] but in most cases, such aromatic residues tend to be rather inert. A highly reactive aromatic cation such as pyrylium as part of the delocalized electron system is an alternative.[1] The reaction of the blue pyrylium label with amines yields a red and fluorescent pyridinium analogue.

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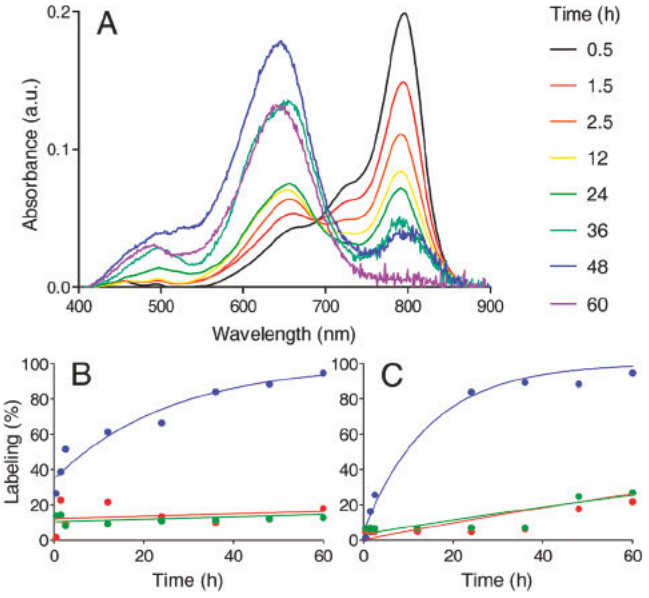

Figure 2. (A) Changing absorbance spectra of dye S 0378 upon binding to HSA at 37 °C. The degree of labeling (%) of HSA (B) and IgG (C) by the dyes S 0121 (red), S 0306 (green), and S 0378 (blue) was calculated according to eq 1. Half times of the labeling kinetics were obtained by fitting the data points to $y = (100 \quad bg) \times (1 \quad e^{-kx}) + bg$, where bg denotes the background and k the reaction rate coefficient. Dye S 0378 displayed the fastest labeling kinetics (half time for labeling HSA, 18 h; and for labeling IgG, 10 h). The reactions of labels S 0121 and S 0306 were too slow to determine accurate half times.

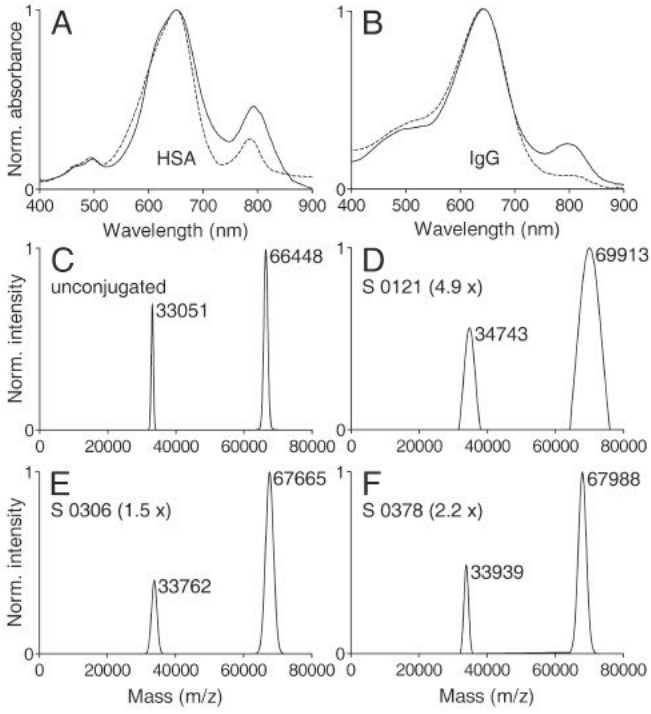

Figure 3. Characterization of labeled proteins. HSA (A) and IgG (B) were labeled with S 0378 and purified by size exclusion chroma tography. An absorbance spectrum was recorded before (solid line) and after (hatched line) purification. Unlabeled and labeled HSA preparations were purified by size exclusion chromatography and analyzed by MALDI TOF mass spectrometry. (C) In the MALDI spectra, the second peak indicates the mass of unconjugated HSA (66.45 kDa (mean) ± 0.57 kDa (standard deviation). (D) HSA labeled with S 0121 (69.91 ± 2.79 kDa). (D) HSA labeled with S 0306 (67.67 ± 1.14). (D) HSA labeled with S 0378 (67.99 ± 1.16). The average number of dye molecules per HSA molecule was calculated by [mass (HSA labeled with dye_x) mass (unconjugated HSA)]/[mass (dye_x) mass (Cl)].

Here, we extend this approach to another kind of cationic π electron system. Cyanine dyes and rhodamines can be regarded as resonance stabilized carbocations that are more electroni cally delocalized than neutral conjugated hydrocarbons.[23] In search of a long wavelength absorbing dye for labeling amines, we found that the chloro atoms at the polymethine chain of cyanine dyes undergo nucleophilic substitution under mild conditions. This particular reactivity places polymethine dyes between polyenes with high chemical reactivity (in terms of addition reactions) and aromatic π systems with their low chemical reactivity (in terms of substitution reactions).[24] Only a few long wavelength absorbing cyanine dyes that contain both a chloro group for nucleophilic substitution by amines and sulfo groups to confer water solubility are available. We chose three exemplary dyes S 0121, S 0306, and S 0378 (the absorbance and emission spectra are shown in the Supporting Information Figure 1) that display a large spectral shift in their absorbance as well as in their emission spectrum when conjugated to amines (Figure 1). Dye S 0306 is a derivative of indocyanine green, which is commonly administered intravenously for medical diagnostics and thus attests to the dye's excellent biocompatibility and nontoxicity.[25]

The chameleon dyes were conjugated to (a) an IgG antibody, (b) human serum albumin (HSA), and (c) the peptide angio tensin II. These compounds can be found in blood and thus represent valuable bioanalytical targets. While labeling of the two proteins is assumed to proceed mainly via their lysine side chains, which typically have a pK_a of 10, the amino terminus of the peptide with its distinctly lower pK_a value is the only conjugation site of the peptide. To account for the high pK_a of lysine side chains, reactions were carried out in bicarbonate buffer of pH 8.4.

Monitoring the Labeling Reaction of Chameleon Dyes. The labeling reaction of all three dyes is visible by eye due to a hypsochromic (shortwave) color shift from green to blue (Figure 1D) as the electron withdrawing chloro group is re placed by an electron donating amino group. The reaction can be monitored quantitatively if the changes in absorbance are recorded over time. The absorbance maxima of the free labels and their conjugates to amines are listed in Figure 1.

A single absorbance measurement, however, is sensitive to the dye concentration as well as the light source and the sensitivity of the sensor. Ratiometric measurements, by contrast, are resistant to such fluctuations but rely on a constant reference value.[26] Although the intensity at the isosbestic point between both maxima should provide a reference value, it is too weak and thus prone to fluctuations. We therefore devised a new ratiometric approach for chameleon dyes based on the absorbance at both maxima, which can be used to report the degree of labeling at any time over the course of the labeling reaction. The sum of the absorbance readings at the absorption maxima of the free dye and the conjugate provide a constant reference value after correcting for the different molar absorption coefficients (listed for each dye in Figure 1; in eq 1, the maxima of label S 0378 are shown). Then, the degree of labeling is defined by the ratio of the absorbance

Table 1. Absorbance at 650 and 800 nm of Dye S 0378 after Reaction with Various Primary Amines[a]

	S 0378 only	β-alanine	dodecylamine	ethanolamine	histamine	propargylamine	tyramine
650 nm	0.03	0.52	0.06	0.35	0.32	0.23	0.33
800 nm	0.59	0.20	0.56	0.00	0.29	0.05	0.02
labeling (%)		82	17	99	66	89	97

[a] The degree of labeling (%) was calculated according to eq 1.

maximum of the conjugate to the reference value.

$$Labeling\ (\%) = \frac{100 \times A_{650\mathrm{nm}}}{A_{650\mathrm{nm}} + A_{800\mathrm{nm}}\left[\varepsilon_{650\mathrm{nm}}(conj)/\varepsilon_{800\mathrm{nm}}(free)\right]} \quad (1)$$

It should be noted that the absorbance of the free dye at 650 nm amounts to about 10% of the absorbance at 800 nm, which changes the degree of labeling slightly. Equation 1 is amenable to any type of chameleon dye as long as there is only a small overlap in the absorbance spectra of the free dye and the conjugate.

Variation of Chameleon Dyes. The labeling of HSA, IgG, and the peptide angiotensin II by the dyes S 0121, S 0306, and S 0378 was monitored by measuring the changes in the absorbance spectra over time. Figure 2A shows the spectra of the reaction of label S 0378 with HSA after correction for stray light. The degree of labeling at each time point was then calculated by eq 1 as shown for HSA (Figure 2B) and IgG (Figure 2C). Similar results were obtained for the peptide. Although all dyes react to some degree with the proteins and the peptide after extended periods of time, only the reaction of dye S 0378 was fast enough to calculate accurate half times for labeling HSA (18 h), IgG (10 h), and angiotensin II (36 h).

The remarkably better labeling kinetics of dye S 0378 can be attributed to the five membered ring at the conjugation site, where the structurally very similar dyes S 0306 and S 0378 feature six membered rings. Both the five membered and the six membered rings favor an S_N1 mechanism over an S_N2 mechanism because the concomitant carbocation is stabilized by the carbon double bonds and the ring structure increases steric hindrance. The five membered ring of dye S 0378, however, shows higher reactivity because cyclopentenyl cations are more stable than cyclohexenyl cations.[27−29] The stabilizing effect of the ring structure thus may also reduce electronic rearrangement along the polymethine chain and retains the carbocation at the site of origin. Figure 1E outlines the inferred reaction mechanism of amines with dye S 0378.

Fluorescence Measurements. In addition to the changes in color, the dyes also exhibit a change in fluorescence upon binding to amines. While the free dyes are weakly fluorescent, the conjugates display deep red fluorescence that can be detected with red sensitive photomultipliers. The reaction of label S 0378 with amines can be monitored by excitation at 600 nm and recording the increase in the emission intensity at 690 nm (Supporting Information Figure 2). Monitoring the labeling reaction by absorbance spectrometry, however, is preferable, because it is ratiometric and thus more precise.

Characterization of Labeled Proteins. IgG and HSA were labeled with dye S 0378 and excess dye was removed by size exclusion chromatography. The fast eluting proteins can be readily distinguished from the unreacted green dye by their blue color. The absorbance at 800 nm shows that some unreacted S 0378 remained noncovalently bound to HSA after the purification step (Figure 3A), while the amount of free dye left on the antibody is negligible (Figure 3B).

Next, the average number of label molecules per protein molecule was determined by MALDI TOF mass spectrometry. All samples were prepared under the same reaction conditions (37 °C; overnight) and purified by size exclusion chromatography. Figure 3C shows the exact molecular mass of HSA with a small standard deviation (66.4 kDa ± 0.6 kDa). The higher molecular masses indicated in Figure 3D−F confirm that the labeling reaction of all three dyes has occurred. HSA was labeled, on average, 4.9 fold by S 0121, 1.5 fold by S 0306, and 2.2 fold by S 0378. The labeled HSA preparations have a broader size distribution than the unconjugated protein because the 58 lysine residues in one HSA molecule can be labeled differentially and to various degrees. The widest distribution of labels on HSA was found with S 0121.

Labeling of Primary Amines. Biogenic and synthetic primary amines (β alanine, dodecylamine, ethanolamine, histamine, propargylamine, and tyramine) were labeled with dye S 0378. These primary amines are less sensitive to solvents such that the labeling reaction can be carried out in methanol. As the primary amines and dyes react in a molar ratio of 1:1, a 2 fold excess of amines was adequate to allow for a complete turnover of the dye molecules. Labeling for 15 h at room temperature (25 °C), however, was only partially successful. Better results were obtained by labeling for 4 h at 70 °C and adding a tertiary amine (triethylamine) as a catalyst and base that cannot react with the dye. In all cases, the reaction can be monitored by the increase in absorbance at 650 nm and the decrease at 800 nm (Table 1). Different primary amines show a remarkably diverse reactivity with dye S 0378. While dodecylamine was only marginally labeled (17%), the reaction was almost complete with ethanolamine (99%) and tyramine (97%).

CONCLUSION

Long wavelength absorbing and fluorescent cyanine dyes were established as a new class of amine reactive chameleon labels for proteins, peptides, and amines. The reactivity of the cyanine dyes is conferred by a chloro substituent in the center of the methine chain that can undergo an S_N1 type nucleophilic substitution with primary amines. Replacement of the chloro substituent results in a visually detectable color change from green to blue as well as a change in fluorescence. A new ratiometric method was devised to study the labeling kinetics. Dye S 0378 showed the fastest kinetics because the reactive chloro group is located at a five membered ring. Although the reaction of dye S 0378 was slower than the reaction of Py labels,[1] proteins and peptides can be easily labeled in aqueous solutions at 37 °C. Even better results can be obtained when the labeling procedure is performed in methanol at 70 °C, which is an additional option for many amines. The outlined reaction mechanism of the long wavelength absorbing cyanine dyes

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holds great potential for the design of future amine reactive chameleon dyes.

■ ASSOCIATED CONTENT

● **Supporting Information.** Absorption and emission spectra of the free dyes and the changes in the fluorescence spectra during the labeling reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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■ REFERENCES

(1) Wetzl, B. K., Yarmoluk, S. M., Craig, D. B., and Wolfbeis, O. S. (2004) Chameleon labels for staining and quantifying proteins. *Angew. Chem., Int. Ed. 43*, 5400 5402.

(2) Craig, D. B., Wetzl, B. K., Duerkop, A., and Wolfbeis, O. S. (2005) Determination of picomolar concentrations of proteins using novel amino reactive chameleon labels and capillary electrophoresis laser induced fluorescence detection. *Electrophoresis 26*, 2208 2213.

(3) Hoefelschweiger, B. K., Duerkop, A., and Wolfbeis, O. S. (2005) Novel type of general protein assay using a chromogenic and fluorogenic amine reactive probe. *Anal. Biochem. 344*, 122 129.

(4) Schneider, E., Keller, M., Brennauer, A., Hoefelschweiger, B. K., Gross, D., Wolfbeis, O. S., Bernhardt, G., and Buschauer, A. (2007) Synthesis and characterization of the first fluorescent nonpeptide NPYY1 receptor antagonist. *ChemBioChem 8*, 1981 1988.

(5) Meier, R. J., Steiner, M. S., Duerkop, A., and Wolfbeis, O. S. (2008) SDS PAGE of proteins using a chameleon type of fluorescent prestain. *Anal. Chem. 80*, 6274 6279.

(6) Wojcik, R., Swearingen, K. E., Dickerson, J. A., Turner, E. H., Ramsay, L. M., and Dovichi, N. J. (2008) Reaction of fluorogenic reagents with proteins I. Mass spectrometric characterization of the reaction with 3 (2 furoyl)quinoline 2 carboxaldehyde, Chromeo P465, and Chromeo P503. *J. Chromatogr., A 1194*, 243 248.

(7) Ramsay, L. M., Dickerson, J. A., Dada, O., and Dovichi, N. J. (2009) Femtomolar concentration detection limit and zeptomole mass detection limit for protein separation by capillary isoelectric focusing and laser induced fluorescence detection. *Anal. Chem. 81*, 1741 1746.

(8) Steiner, M. S., Meier, R. J., Spangler, C., Duerkop, A., and Wolfbeis, O. S. (2009) Determination of biogenic amines by capillary electrophoresis using a chameleon type of fluorescent stain. *Microchim. Acta 167*, 259 266.

(9) Cohen, D., Dickerson, J. A., Whitmore, C. D., Turner, E. H., Palcic, M. M., Hindsgaul, O., and Dovichi, N. J. (2008) Chemical cytometry: Fluorescence based single cell analysis. *Annu. Rev. Anal. Chem. 1*, 165 190.

(10) Hoffmann, K., Mix, R., Resch Genger, U., and Friedrich, J. F. (2007) Monitoring of amino functionalities on plasma chemically modified polypropylene supports with a chromogenic and fluorogenic pyrylium reporter. *Langmuir 23*, 8411 8416.

(11) Steiner, M. S., Meier, R. J., Duerkop, A., and Wolfbeis, O. S. (2010) Chromogenic sensing of biogenic amines using a chameleon probe and the red green blue readout of digital camera images. *Anal. Chem. 82*, 8402 8405.

(12) Garcia Acosta, B., Comes, M., Bricks, J. L., Kudinova, M. A., Kurdyukov, V. V., Tolmachev, A. I., Descalzo, A. B., Marcos, M. D., Martinez Manez, R., Moreno, A., Sancenon, F., Soto, J., Villaescusa, L. A., Rurack, K., Barat, J. M., Escriche, I., and Amoros, P. (2006) Sensory hybrid host materials for the selective chromo fluorogenic detection of biogenic amines. *Chem. Commun.* 2239 2241.

(13) Kostenko, O. M., Kovalska, V. B., Volkova, K. D., Shaytanov, P., Kocheshev, I. O., Slominskiy, Y. L., Pisareva, I. V., and Yarmoluk, S. M. (2006) New method for covalent fluorescent biomolecule labeling with hemicyanine dye. *J. Fluoresc. 16*, 589 593.

(14) Miller, J. N. (2008) *Long wavelength and near infrared fluorescence: State of the Art, Future applications, and Standards*, Springer, Berlin.

(15) Oswald, B., Patsenker, L., Duschl, J., Szmacinski, H., Wolfbeis, O. S., and Terpetschnig, E. (1999) Synthesis, spectral properties, and detection limits of reactive squaraine dyes, a new class of diode laser compatible fluorescent protein labels. *Bioconjugate Chem. 10*, 925 931.

(16) Oswald, B., Gruber, M., Bohmer, M., Lehman, F., Probst, M., and Wolfbeis, O. S. (2001) Novel diode laser compatible fluorophores and their application to single molecule detection, protein labeling and fluorescence resonance energy transfer immunoassay. *Photochem. Photobiol. 74*, 237 245.

(17) Pham, W., Lai, W. F., Weissleder, R., and Tung, C. H. (2003) High efficiency synthesis of a bioconjugatable near infrared fluorochrome. *Bioconjugate Chem. 14*, 1048 1051.

(18) Patonay, G., Salon, J., Sowell, J., and Strekowski, L. (2004) Noncovalent labeling of biomolecules with red and near infrared dyes. *Molecules 9*, 40 49.

(19) Kele, P., Li, X. H., Link, M., Nagy, K., Herner, A., Lorincz, K., Beni, S., and Wolfbeis, O. S. (2009) Clickable fluorophores for biological labeling with or without copper. *Org. Biomol. Chem. 7*, 3486 3490.

(20) Li, X. H., Shi, W., Chen, S. M., Jia, J., Ma, H. M., and Wolfbeis, O. S. (2010) A near infrared fluorescent probe for monitoring tyrosinase activity. *Chem. Commun. 46*, 2560 2562.

(21) Zollinger, H. (2003) *Color chemistry, synthesis properties, and applications of organic dyes and pigments*, 3rd ed., Wiley VCH, Weinheim, Germany.

(22) Ghosh, P. B., and Whitehou, Mw. (1968) 7 chloro 4 nitrobenzo 2 oxa 1,3 diazole a new fluorigenic reagent for amino acids and other amines. *Biochem. J. 108*, 155 156.

(23) Thorley, K. J., Hales, J. M., Anderson, H. L., and Perry, J. W. (2008) Porphyrin dimer carbocations with strong near infrared absorption and third order optical nonlinearity. *Angew. Chem., Int. Ed. 47*, 7095 7098.

(24) Reichardt, C. (1995) Chiral polymethine dyes: A remarkable but forgotten conjugated pi system. *J. Phys. Org. Chem. 8*, 761 773.

(25) Landsman, M. L. J., Kwant, G., Mook, G. A., and Zijlstra, W. G. (1976) Light absorbing properties, stability, and spectral stabilization of indocyanine green. *J. Appl. Physiol. 40*, 575 583.

(26) Gorris, H. H., Ali, R., Saleh, S. M., and Wolfbeis, O. S. (2011) Tuning the dual emission of photon upconverting nanoparticles for ratiometric multiplexed encoding. *Adv. Mater. 23*, 1652 1655.

(27) Brown, H. C., Rao, C. G., and Ravindranathan, M. (1978) Structural effects in solvolytic reactions 0.29. Solvolysis of tertiary allylic para nitrobenzoates Effect of allylic double bond on rates of solvolysis of representative tertiary para nitrobenzoates. *J. Org. Chem. 43*, 4939 4943.

(28) Schleyer, P. V., Bentley, T. W., Koch, W., Kos, A. J., and Schwarz, H. (1987) Structures and energies of isomeric cyclopentenyl cations. Resolution of the question of anchimeric assistance in cyclopenten 4 yl solvolysis. *J. Am. Chem. Soc. 109*, 6953 6957.

(29) Sorensen, T. S. (1969) The equilibria and kinetics of the cyclohexenyl cyclopentenyl cation rearrangement. *J. Am. Chem. Soc. 91*, 6398 6403.