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Syntheses of Novel Dipyrrylmethene-BF_2 Dyes and Their Performance as Labels in Two-Photon Excited Fluoroimmunoassay

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Two-photon excitation of fluorescence (TPE) has been found a powerful tool in the field of microscopy imaging and recently also in the field of bioanalytics. The recently introduced bioaffinity assay technology, ArcDia™ TPX, enables separation-free ultra-sensitive immunoassays from microvolumes. This assay technique is based on the use of microspheres as a solid reaction carriers and two-photon excited fluorescence detection. In the ArcDia™ TPX-technology, the individual microparticles are observed and the number of bound biomolecules on the microparticle surface is quantified by two-photon excited fluorescence. Here we present synthesis and use of a novel dipyrrylmethene-BF_2 fluorophore that has been designed to be used as label in ArcDia™ TPX assay technique. The absorption and emission wavelengths of the label are tuned to allow excitation with a 1064 nm microchip laser. The label contains two-carboxylic residues, one of which is activated as N-hydroxysuccinimide ester to enable labeling of amino residues of biomolecules. The other carboxylic group is in free form to increase solubility in aqueous solutions. This new fluorescent label is tested in a separation-free immunoassay using ArcDia™ TPX assay technique. The performance of the new label is compared to that of one of the brightest fluorophores available, *R*-phycoerythrin (RPE). According to the results, the dipyrrylmethene-BF_2 label provides significantly better signal-to-background ratio, leading to higher assay sensitivity and broader dynamic range compared to that of RPE. Good solubility to aqueous solutions and high fluorescence quantum efficiency, suggests the dipyrrylmethene-BF_2 label is applicable also in other fluorescence-based applications.

KEY WORDS: Two-photon excited fluorescence; dipyrrylmethene-BF_2; BODIPY; homogeneous; immunoassay; labeling reagant.

INTRODUCTION

The usefulness of two-photon excitation of fluorescence (TPE) in laser scanning microscopy was first realised by Denk *et al.* [1]. The introduction of Ti:sapphire lasers facilitated the implementation of TPE in a standard laser scanning fluorescence microscopy [2]. The developments have also lead to industrial manufacture of two-photon laser scanning microscope systems and nowadays TPE microscopy can be considered as a routine tool in the field of microscopy imaging [3].

One of the inherent features that relate to TPE techniques is the relatively low signal level that originates from the small excitation volume. Besides that, the low signal level can originate from low two-photon absorption cross-section of fluorophores or from low excitation rate. These are naturally dependent on the properties of the illumination source used for excitation. Large part of the research work in the area of TPE has been carried out using mode locked femtosecond lasers [4–7]. These lasers are capable to generate short pulses with a high repetition frequency. The advantage of this type of lasers is that they provide

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high excitation rate. The major disadvantage of the mode-locked femtosecond lasers is their high cost and complicated operation routines. Therefore these lasers are mainly used for scientific purposes.

The recent development of less expensive lasers is very encouraging in regard to usefulness TPE in routine bioanalytics. We have recently shown that TPE provides a powerful tool for monitoring bioaffinity reactions in microvolumes [8–11]. This novel ArcDia^{TM} TPX assay technique is based on use of microparticles as a solid phase to bind the biomolecules to be studied. Each microparticle acts as a local concentrator of the biomolecules. When a fluorescent biospecific tracer molecule attaches to the microparticle, the degree of binding becomes measurable by observing the TPE signal from individual microparticles. This assay technique is separation free, ie. no washing steps are needed. The instrument used in the assay technique includes microchip Nd:YAG laser that generates nanosecond pulses with ~20 kHz repetition rate at wavelength of 1064 nm. Currently Nd:YAG microchip lasers are the only possible lasers, in terms of cost and excitation efficiency, that can be used for TPE in the instrument designed for routine bioanalytical applications [12]. Although, this laser and the ArcDia^{TM} TPX technique can be used in combination with many conventional fluorescent labels, development of new two-photon excitable labels is necessary in order to reach maximum assay performance.

In the field of TPE microscopy the development of the two-photon excitable fluorophores has mainly focused on maximising of two-photon excitation cross-section [13–18]. Compared to mode-locked femtosecond lasers used in the field of TPE microscopy, the microchip lasers currently used in the ArcDia^{TM} TPX microfluorometer suffer from low repetition rate. However, due to the high peak power of these lasers, the excitation efficiency per single laser pulse is generally high enough to excite fluorophores even with moderate two-photon excitation cross-section. Therefore, increasing the cross-section is not the key objective in the development of fluorophores for ArcDia^{TM} TPX-technique. In some cases, large cross section together with high peak power laser may even give rise to undesired phenomena, like stimulated emission or excited state absorption, that can reduce final obtainable TPE emission output.

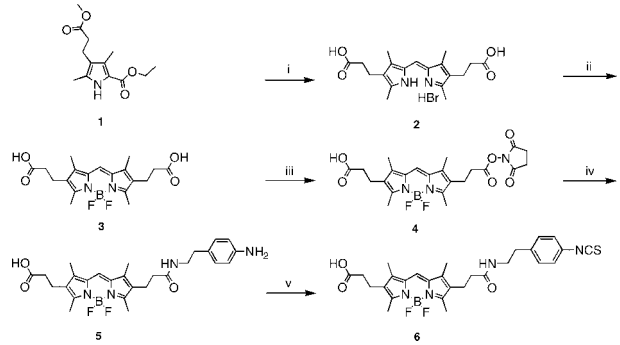
The dipyrrylmetheneboron difluoride dyes (dipyrrylmethene-BF_2 dyes) were introduced by Treibs and Kreuzer in the 1960's [19]. Since then, dipyrrylmethene-BF_2 dyes have found various applications. A wide variety of dipyrrylmethene-BF_2 dyes are commercially available and are sold under trademark BODIPY® [20]. These fluorescent dyes have been used as tracers in fluorescence microscopy, in fluorescence immunoassay and in flow cytometric analysis. So far, only little attention has been paid to the two-photon absorption and emission properties of these dyes [5, 21]. As fluorescent label compounds these dyes have many desirable properties: High quantum efficiency, sharp absorption and emission bands and relatively high absorption coefficient. Most of the dipyrrylmethene-BF_2 dyes, however, suffer from intrinsic hydrophobicity that limits their use in fluorescent labeling of biomolecules.

In this paper we describe synthesis of a novel hydrophilic labeling reagent that is based on dipyrrylmethene-BF_2 fluorophore. The fluorophore was designed to be excitable with Nd:YAG laser generating light of 1064 nm. Two-photon excitation cross-section for the fluorophore was determined relative to a reference compound reported earlier in the literature. The performance of the new dipyrrylmethene-BF_2 label is compared to that of one of the brightest fluorophores available, R-phycoerythrin (RPE), in a separation-free immunoassay using the ArcDia^{TM} TPX assay technique.

RESULTS AND DISCUSSION

Synthesis

The new dipyrrylmethene-BF_2 dyes were synthesised by using 2-ethoxycarbonyl-3,5-dimethyl-4-methoxycarbonylethylpyrrole (**1**) [22] as a starting material (Scheme 1.). The first step was formation of dipyrrylmethene (**2**) in a solution of hydrobromic and formic acid. The use of a mixture of hydrobromic and formic acid in syntheses of dipyrrylmethenes was first described by Fischer *et al.* [23] in synthesis of coproporphyrins and mesoporphyrins. The reaction is simple to perform, utilises only one pyrrole as a starting material,



Scheme 1. Synthesis of dipyrrylmethene-BF_2 labels (i) HBr—H_2O, HCOOH, 100°C, 2 hr (ii) TEA, $BF_3^*O(Et)_2$, $CHCl_3$, RT, 2.5 hr (iii) NHS, DCC, DMF, RT, 24 hr (iv) aminoethylaniline, TEA, DMF, RT, 30 min (v) $CSCl_2$, $CHCl_3$, $NaHCO_3$, RT, 3 min.

and affords dipyrrylmethene **2** in high yield. The product, dipyrrylmethene **2**, crystallised out from the cooled reaction mixture as small, orange, needle like crystals. The dipyrrylmethene **2** was dissolved in a mixture of chloroform and triethylamine, and complexed with boron trifluoride ethyl etherate to give compound **3**. Formation of the boron complex (**3**) was observed immediately after addition of boron trifluoride ethyl etherate from green-yellow fluorescence of the reaction mixture. The reaction mixture was diluted with chloroform and washed with dilute hydrochloric acid to remove small amount of unreacted starting material. The product (**3**) was further purified by precipitation from ethanol/water to give compound **3** as orange-brown powder in high yield. One of the two carboxylic acid residues was transformed to succinimidyl ester by treatment with 1 equivalent *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 3 equivalents of *N*-hydroxysuccinimide (NHS) in dry DMF. DCC was used as a limiting component in the reaction to minimise the formation of the diester by-product. NHS was used in excess to ensure fast conversion of the activated carboxylic acid to corresponding succinimidyl ester and thus to minimise the formation of acylurea by-product. The desired mono-activated dipyrrylmethene-BF_2 (**4**) was obtained after column chromatography on silica gel in 39% yield. By recycling the recovered starting material the yield can be somewhat improved. The small amount of disuccinimidyl ester that is formed in the reaction can also be recycled. The hydrolysis of the disuccinimidyl ester can be easily performed in slightly alkaline conditions (pH < 10). Higher pH values should be avoided since it results in decomposition of the boron complex. After the recycling procedures the total yield of dipyrrylmethene-BF_2 (**4**) increases up to 60%.

The succinimidyl ester (**4**) was used for labeling experiments and as a starting material for synthesis of aminoethylaniline derivative (**5**). The reaction with aminoethylaniline was performed in anhydrous DMF and proceeded with almost quantitative yield. The aminoethylaniline derivative (**5**) was reacted with thiophosgene in chloroform-bicarbonate suspension in order to obtain isothiocyanate derivative (**6**). The synthetic products (**2**–**6**) were characterized by using ^{1}H NMR and MS techniques.

Mass Spectrometry

The mass spectrometry was performed by monitoring both negative and positive ions. The molecular peaks in positive mode corresponded either M^+ or MH^+ ions. Molecular peak of the dipyrrylmethene **2** and the boron complexes **5** and **6** were observed in a form of MH^+ ion whereas the boron complexes **3** and **4** were observed in form of M^+ ion. All the boron complexes **3**–**6** show, besides the molecular peak, also a peak that corresponded to a fragment with a molecular weight of MH^+-19 or M^+-19. This corresponds to a molecular fragment that misses one fluorine ion. Usually the intensity of the fragment MH^+-19 or M^+-19 peak was larger than the intensity of the molecular peak. The succinimidyl ester (**4**) was analysed also with MS-FAB and MS-ES techniques, which also gave molecular peaks that corresponded to the M^+ ion. However, in MS-ES besides the M^+ peak also the MH^+ peak was present although in small intensity. In negative mode the molecular peaks could not be detected either with MS-MALDI or MS-FAB techniques. With MS-ES the molecular peak of the negative M-H ion was easily detected.

Labeling of Immunoglobulin G

Performance of the aminoreactive labeling reagents **4** and **6** were tested in labeling of monoclonal mouse IgG. Both labeling reagents were used in 10 fold molar excess with respect to the IgG. The succinimidyl ester derivative (**4**) was incubated for 2 hr with IgG in slightly alkaline pH (8.3). Higher pH (9.3) and longer incubation time (24 hr) was used for isothiocyanate derivative (**6**) because of the lower reactivity of isothiocyanato moiety compared to succinimidyl ester. Both reaction mixtures were purified with gelfiltration chromatography. The purity was checked with HPLC using superdex 200 gelfiltration column. The label-IgG conjugates eluted at 27 min while no free label was observed. The labeling degrees were determined spectrophotometrically. With succinimidyl derivative **4** a labeling degree of 6 fluorophores per IgG and with isothiocyanate derivative **6** a labeling degree of 3.4 fluorophores per IgG was obtained. According to these results the succinimidyl ester derivative seems to provide more efficient labeling than the isothiocyanate derivative. However, the concentration of the IgG was lower in case of isothiocyanate and this will obviously lower the labeling degree. The pH in the labeling reaction with the isothiocyanate label could be raised to reach optimum conditions for this reactive group and thus to increase the reaction rate. However, increase in pH could also lead to decomposition of the boron complex and therefore relatively low pH was used here.

The IgG-*R*-phycoerythrin conjugate was prepared according to typical cross-linking procedure. The product was purified with gelfiltration chromatography using Superdex 200 column. The product (1:1 conjugate) eluted from the column between 21 to 23 min. (peak at 22 min) whereas retention time for the unreacted RPE was 25 and for the IgG 27 min.

Table I. Absorption and Fluorescence Data

Dye	$\lambda_{Abs,MeOH}$ (nm)	ε_{MeOH} ($cm^{-1}M^{-1}$)	$\lambda_{fluor,MeOH}$ (nm)	$\phi_{fluor,MeOH}$
3	526	74,000	533	0.90
4	524	75,000	533	0.91
5	525	72,000	533	0.24
5 (H^+)	524	73,000	533	0.79
6	526	75,000	533	0.84

Note. Fluorescence spectra were measured from 125 nM solutions using halogen lamp illumination at 480 nm.

Absorption and Fluorescence Spectroscopy

Absorption and emission spectra of the dipyrrylmethene-BF_2 dyes **3**–**6** were measured in methanol. The results are summarised in Table I. The absorption and emission spectra of the dipyrrylmethene-BF_2 succinimidyl ester (**4**) is presented in Fig. 1. The dipyrrylmethene-BF_2 dyes **3**–**6** showed sharp absorption peak around 525 nm in methanol solution. All the fluorophore derivatives **3**–**6** exhibited emission maximum at 533 nm. The absorption and emission properties of dipyrrylmethene-BF_2 dyes are generally considered to be rather insensitive to environment [20]. As expected, change of the solvent from methanol to phosphate buffer did not cause remarkable shifts on absorption or emission maximum and neither in shape of the spectra. However, the absorption coefficient decreased from 75,000 to 68,000 cm^{-1} M^{-1} and also the fluorescence quantum yield decreased from 0.91 to 0.78. The dipyrrylmethene-BF_2 dye **5** showed remarkably lower fluorescence quantum yield than the dyes **3**, **4** and **6**.

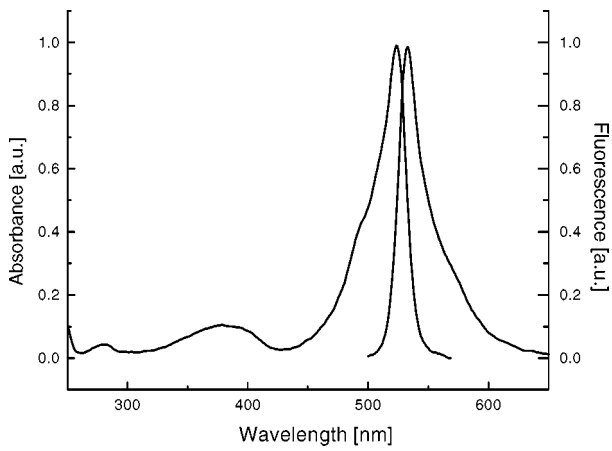


Fig. 1. Absorption and emission spectrum of dipyrrylmethene-BF_2 succinimidyl ester (**4**) in methanol. Fluorescence spectrum was measured using halogen lamp illumination source (480 nm).

The dipyrrylmethene-BF_2 dye **3** showed only a limited solubility to methanol and upon standing dye aggregates are formed leading to remarkable decrease in quantum yield. Therefore, the measurement of fluorescence quantum yield of compound **3** was performed immediately after dilution with methanol. The low quantum yield of compound **5** can be explained by intramolecular electron transfer process between the aniline moiety and the BF_2 chromophore. Such intramolecular electron transfer was reported by Kollmannsberger *et al.* [24] in the BF_2 chromophore where the *N*,*N*-dimethylaniline was directly attached to the π-electron system of the BF_2 chromophore. In order to study the reason for the decrease of the quantum yield of compound **5**, we titrated the methanol solution of the compound **5** with hydrochloric acid. As a result the fluorescence intensity and the determined quantum yield increased dramatically from 0.24 up to 0.79. In protonated form the aniline moiety cannot act as an electron donor and thus the intramolecular electron transfer process is prohibited.The IgG-label-conjugates showed equal spectra with those of the free labels. The main absorption band around 500 nm was slightly broader than the absorption bands of the free labels. The fluorescence intensity was however quite dramatically decreased. The fluorescence yield per label (at labeling degree 6 labels per protein) was only 16% of the fluorescence yield obtained from the free label. This conjugation related quenching is strongly dependent from the labeling degree and can be reduced by lowering the labeling degree. However, according to Poisson statistics, lowering the labeling degree also increases the proportion of the unlabeled IgG. When considering the assay performance, large proportion of unlabeled IgG in the assay would decrease the assay sensitivity. The effect of labeling degree on the performance of immunometric assay using ArcDia^{TM} TPX assay technique will be discussed and published elsewhere.

The two-photon excited fluorescence was measured using ArcDia^{TM} TPX microfluorometer [25]. The instrument was equipped with an emission filter that enables signal collection in the range of 530–610 nm. The two-photon excited fluorescence yields of the labeling reagents **4** and **6**, and the corresponding IgG conjugates are presented in Table II. The two-photon excitation cross section was determined to be in order of 2×10^{-49} cm^4s/photon (1064 nm) by comparing the fluorescence signal of the dipyrrylmethene-BF_2 succinimidyl ester (**4**) to the fluorescence signal of RPE [26]. This value is in good agreement to the value reported earlier by Xu and Webb for another dipyrrylmethene-BF_2 dye [5]. Recently Bestwater F. *et al.* [21] reported TPE spectra for two dipyrrylmethene-BF_2 dyes having one-photon absorption maxima at 505 nm and

Table II. Two-Photon Excited Fluorescence Intensities of Dipyrrylmethene-BF_2 Labels and IgG Conjugates

Sample	TPE (a.u.)
Label **4**	87
Label **6**	100
IgG-label **4** conjugate	86
IgG-label **6** conjugate	53
IgG-*R*-phycoerythrin conjugate	1510

Note. Free labels **4** and **6** are measured in methanol (125 nM) and the IgG conjugates are measured in PBS (125 nM in respect to the IgG). Excitation with Nd:YAG laser.

589 nm and emission maxima at 511 and 617 nm respectively. Their research related to microscopic imaging and was more qualitative than quantitative in nature. In that paper TPE cross-section values for dipyrrylmethene-BF_2 dyes were not reported.

Separation Free Immunoassay

Performance of the IgG-label-conjugates was tested in a separation-free immunoassay using ArcDia^{TM} TPX assay technique. In this technique, the analyte molecules are recognized and bound by two antibody components, which are: 1) primary antibody coated on the surface of polystyrene microparticles and 2) secondary antibody conjugated with a fluorescent label. Consequently, fluorescent sandwich complexes are formed on the surface of microparticles. The fluorescence from individual microparticles are then measured, separation free, with a ArcDia^{TM} TPX microfluorometer.

In this study the model assay was an immunometric assay of hAFP. The microparticles are coated with a specific antibody against hAFP. The monoclonal mouse IgG against another epitope of hAFP, labeled either with dipyrrylmethene-BF_2 succinimidyl ester (**4**) or with *R*-phycoerythrin, was used as a tracer. The reaction mixtures were incubated 24 hr prior to measurements to ensure equilibrium between bound and free fraction. The reaction mixtures were measured with two ArcDia^{TM} TPX instruments. One was equipped with Nd:YAG microchip laser with a nominal pulse length of 1 ns, pulse repetition rate of 17 kHz and average power of 70 mW, and another was equipped with a mode-locked femtosecond diode pumped Nd:Glass laser with a pulse length of 140 fs, repetition rate of 110 MHz and an average power of 150 mW. The assay data obtained with Nd:YAG laser (Figs. 2 and 3) shows clear difference in assay performance between the RPE and dipyrrylmethene-BF_2 label. As expected, based on the

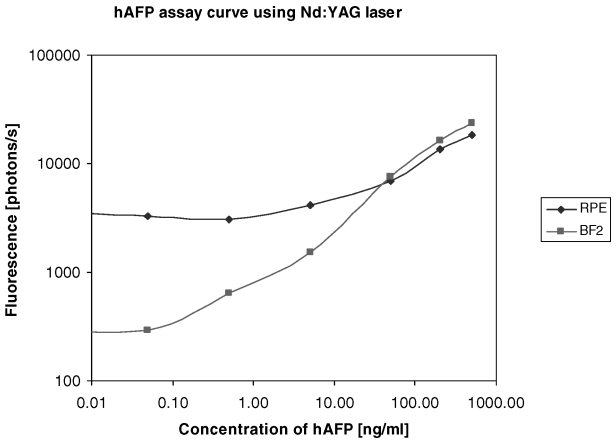


Fig. 2. hAFP assay curves obtained with ArcDia^{TM} TPX instrument equipped with Nd:YAG laser.

solution measurements (Table II), TPE signal of the zero AFP standard (background signal) from the assay of the RPE tracer is more than order of magnitude higher than the corresponding signal of dipyrrylmethene-BF_2 tracer. The standard curve for hAFP with dipyrrylmethene-BF_2 tracer show expected signal response. Instead, the TPE signal obtained from the RPE tracer is not increasing as rapidly as expected. Compared to dipyrrylmethene-BF_2 tracer the difference between these labels can be seen clearly in Figs. 2 and 3. The background-subtracted signals of the dipyrrylmethene-BF_2 conjugate are even higher than the signals obtained from the RPE conjugate, which is a surprise when taking into account the solution measurements. This phenomenon can be partly explained by saturation of the dye molecules due to the high excitation intensity. However, it seems that the emission of RPE conjugate on the surface of the microparticles is also

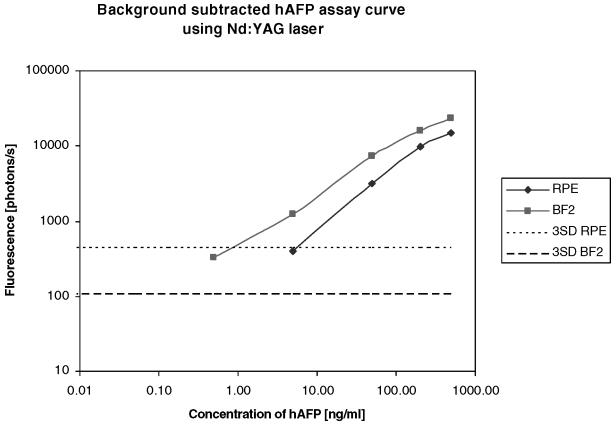


Fig. 3. Background subtracted hAFP assay curves and 3SD levels obtained with ArcDia^{TM} TPX instrument equipped with Nd:YAG laser.

somewhat quenched. This is because the specific signal (background subtracted signal) from the RPE remains lower than the signal of dipyrrylmethene-BF_2 throughout the assay range. The origin of this quenching however remains unclear. It has been reported in the literature that RPE possesses quite remarkable photobleaching under two-photon excitation [27]. Since in the ArcDia^{TM} TPX assay technique each microparticle is measured few milliseconds, besides the saturation also photobleaching could be an explanation for the low signal levels obtained with RPE conjugate. Also excited-state absorption or stimulated emission could be an explanation for the behaviour of RPE on the surface of microparticles. Such explanation has been suggested earlier [28] for explaining the abnormal behaviour of Rhodamine B under two-photon excitation.

The same reaction mixtures were measured also with an ArcDia^{TM} TPX instrument that was equipped with a Nd:Glass laser. The results are summarised in Figs. 4 and 5. The dipyrrylmethene-BF_2 conjugate provides also with the Nd:Glass laser an appropriate assay curve with high signal-to-background ratio. In this case, however, the RPE conjugate provides more TPE-signal than the dipyrrylmethene-BF_2 conjugate, also when the background is subtracted. The higher signal obtained with RPE conjugate also from the surface of the microparticles suggests, that with proper assay optimisation, as sensitive or even more sensitive assay can be obtained by using RPE as a fluorescent label instead of dipyrrylmethene-BF_2. This is the case especially if there is no need for large dynamic range in the assay. Compared to the results obtained with Nd:YAG laser the TPE-signal levels (photons/s) for both tracers are about order of magnitude

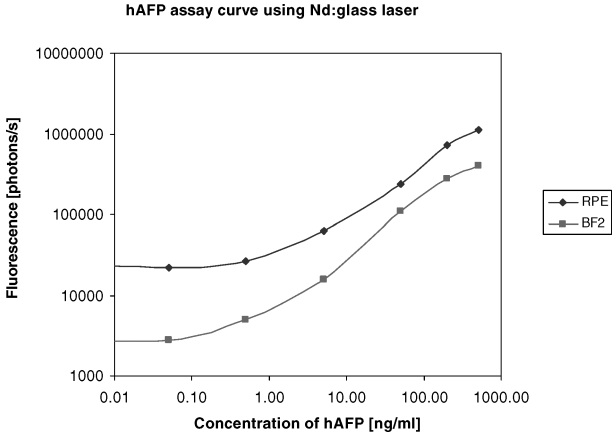


Fig. 4. hAFP assay curves obtained with ArcDia^{TM} TPX instrument equipped with Nd:glass laser.

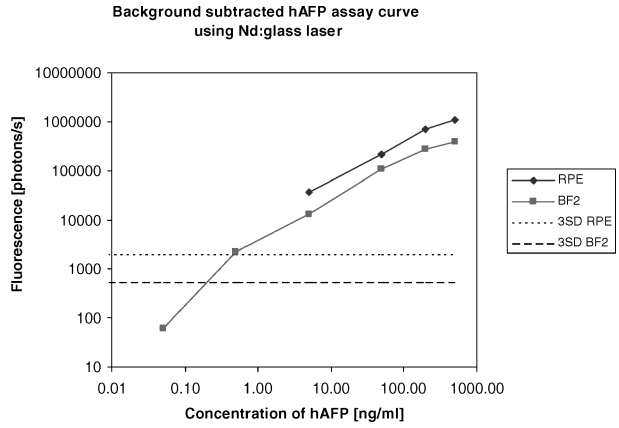


Fig. 5. Background subtracted hAFP assay curves and 3SD levels obtained with ArcDia^{TM} TPX instrument equipped with Nd:Glass laser.

higher. This is mostly due to the high repetition rate of the Nd:Glass laser.

EXPERIMENTAL

Materials

The reagents for syntheses were purchased either from Fluka, J.T. Baker or Merck and used without further purification. The solvents were p.a. grade either from J.T. Baker, Lab-Scan or Merck and used as received unless otherwise stated. The *R*-phycoerythrin-SPDP conjugate was purchased from Molecular Probes and the bifunctional linker GMBS was from Pierce Chemical Company. Monoclonal mouse IgG against human α-fetoprotein (hAFP, clone 5108) was purchased from Medix Biochemica (Finland) and the hAFP standard was form Dako AS (X0900, Dako A/S, Denmark). The polystyrene microparticles were purchased from Bangs Laboratories (carboxy modified microspheres, PC05N, Fishers, IN). ^{1}H NMR spectra were recorded either on JEOL JNM-LA400 or Bruker Avance DRX 500 spectrometer using deuterated chloroform (containing 5% deuterated methanol), or dimethylsulfoxide as a solvent and tetramethylsilane as internal standard. Mass spectra (MS) were recorded either on a Voyager DE-PRO MALDI TOF (Perseptive Biosystems) using α-cyano-4-hydroxy-cinnamic acid as matrix, or on Mariner ESI-TOF (Perseptive Biosystems) or on ZABSpec-oaTOF (Fisons Instruments). The UV–Vis spectra were recorded on a SD-2000 Ocean Optics single beam fiber optic diode array spectrophotometer. Thin-layer chromatography (TLC) plates (Si 60 F_{254}) and silica gel 60 used for absorption chromatography were obtained from Merck. The

NAP-5 and the Superdex-200 gelfiltration columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Synthesis

*3,3′,5,5′-Tetramethyl-4,4′-carboxyethyl-2,2′-dipyrrylmethene hydrobromide (**2**)*

2-Ethoxycarbonyl-3,5-dimethyl-4-methoxycarbonylethylpyrrole (**1**) (1.0 g, 3.35 mmol) was dissolved in formic acid (3 ml) and hydrobromic acid (48%, 3 ml) was added. The reaction mixture was stirred at 100°C for 2.5 hr, and left standing at room temperature overnight. The crystalline product was filtrated off and washed with water (20 ml). The product (**2**) was dried in vacuum desiccator over silica gel. The yield was 677 mg (84%). Additional amount of crystalline product (43 mg) was obtained from the filtrate after standing two days at +4°C. The total yield was 720 mg (86%).

MS (MALDI): found 345 (M + H), calculated 345 (M + H).

^{1}H NMR (Bruker Avance DRX 500, DMSO-d_6, 500 MHz, δ ppm): 7.17 (1H, s, Ar—CH═), 2.65 (4H, t, 2*x*—CH_2), 2.43 (4H, m, 2*x*—CH_2—), 2.40 (6H, s, 2*x*—CH_3), 2.25 (6H, s, 2*x*—CH_3).

*4,4-Difluoro-1,3,5,7-tetramethyl-2,6-di-(2-carboxyethyl)-4-bora-3a,4a-diaza-s-indacene (**3**)*

Dipyrrylmethene hydrobromide (**2**) (500 mg, 1.17 mmol) was suspended in chloroform (20 ml) and triethylamine (4.3 ml, 31 mmol) was added. The starting dipyrrylmethene (**2**) dissolved immediately after triethylamine addition. Boron trifluoride ethyletherate (5 ml, 31 mmol) was added. Reaction mixture was stirred at room temperature for 2.5 hr. The reaction mixture was diluted with chloroform (50 ml), washed with HCl (5% aq, 30 ml) and water (30 ml). Chloroform phase was evaporated to dryness and the product (**3**) was crystallised from aqueous ethanol (50%, 7 days at +4°C) yielding brown-orange powder. The product was dried in vacuum desiccator over silica gel to yield 420 mg (92%) of compound **3.**

^{1}H NMR (JEOL JNM-LA400, DMSO-d_6, 400 MHz, δ ppm): 7.53 (1H, s, Ar—CH═), 2.57 (4H, t, 2*x*-CH_2), 2.38 (6H, s, 2*x*-CH_3), 2.29 (4H, t, 2*x*-CH_2—), 2.19 (6H, s, 2*x*-CH_3).

MS (MALDI): found 392 (M^+), 373 ($M^+ - 19$), calculated 392 (M^+).

UV–Vis (MeOH): $\lambda_{max} = 523$ nm ($\varepsilon = 74,000$ $cm^{-1}M^{-1}$).

*4,4-Difluoro-1,3,5,7-tetramethyl-2-(2-succinimidoxycarbonylethyl)-6-carboxyethyl-4-bora-3a,4a-diaza-s-indacene **(4)***

Compound (**3**) (102 mg, 0.26 mmol) was dissolved in anhydrous DMF (2.5 ml). *N*-hydroxysuccinimide (90 mg, 0.78 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (54 mg, 0.26 mmol) were added. Reaction mixture was stirred for 24 hr at room temperature. The urea precipitate was filtrated off and DMF was evaporated (5 mbar, 40–50°C). The product was purified with column chromatography on silica gel using dichloromethane:acetone:acetic acid (100:8:1, v:v:v) as eluent. Fractions containing the mono-succinimidyl ester (**4**) were combined and evaporated to dryness. The product was precipitated from dichloromethane with petroleum ether, filtered off and dried under atmosphere to give compound **4** as an orange solid. Yield 50 mg (39%).

^{1}H NMR (JEOL JNM-LA400, DMSO-d_6, 400 MHz, δ ppm): 7.60 (1H, s, Ar—CH═), 2.85 (2H, t, —CH_2—), 2.80 (4H, s, 2*x*-CH_2—), 2.72 (2H, t, —CH_2—), 2.59 (2H, t, —CH_2—), 2.41 (3H, s, CH_3), 2.40 (3H, s, CH_3), 2.36 (2H, t, —CH_2—), 2.22 (3H, s, CH_3), 2.21(3H, s, CH_3).

MS (MALDI,): found 489 (M^+), 470 ($M^+ - 19$), calculated 489 (M^+).

MS (FAB): found 489(M^+), 470 ($M^+ - 19$), calculated 489 (M^+).

MS (ES): Positive ions: found 512 (M + Na), 490 (M + H), 489 (M^+), 470 ($M^+ - 19$), calculated 489 (M^+); Negative ions: found 488 (M − H), calculated 488 (M − H).

UV–Vis (MeOH): $\lambda_{max} = 524$ nm ($\varepsilon = 75,000$ $cm^{-1}M^{-1}$).

*4,4-Difluoro-1,3,5,7-tetramethyl-6-carboxyethyl-2-[2-(4-aminophenyl)ethylcarbamylethyl]-4-bora-3a,4a-diaza-s-indacene **(5)***

Compound (**4**) (47 mg, 96 μmol) was dissolved in anhydrous DMF (1 ml). Triethylamine (40 μl, 288 μmol) and aminoethylaniline (19.6 mg, 144 μmol) in DMF (1 ml) were added. The reaction mixture was stirred for 30 min at room temperature, diluted with dichloromethane (50 ml), washed with HCl (5% aq, 30 ml) and water (3 × 30 ml). Organic phase was dried with anhydrous Na_2SO_4, filtrated and evaporated to dryness. The product (**5**) was precipitated from dichloromethane with carbon tetrachloride, and dried in vacuum desiccator over silica gel. This gave compound **5** as brown-orange powder in 98% yield (48 mg).

^{1}H NMR (Bruker Avance DRX 500, $CDCl_3$ (5% CD_3OD), 500 MHz, δ ppm): 6.96 (1H, s, Ar—CH═), 6.79 and 6.55 (4H, 2d, $J = 7.6$ Hz, Ph), 5.32 (1H,

bs, —NH—), 3.41 (2H, q, $J = 12.6$ Hz, —CH_2—), 2.74 (2H, t, $J = 7.2$ Hz, —CH_2—), 2.70 (2H, t, $J = 7.3$ Hz, —-CH_2—), 2.61 (2H, t, $J = 5.8$ Hz, —CH_2—), 2.52 (3H, s, CH_3), 2.50 (2H, t, shielded by methyl peaks, —CH_2—), 2.47 (3H, s, CH_3), 2.21 (2H, t, shielded by methyl peaks, —CH_2—), 2.20 (3H, s, CH_3), 2.15 (3H, s, CH_3).

MS (MALDI): found 511 (M + H), 492 (M + H − 19), calculated 511 (M + H).

UV–Vis (MeOH): $\lambda_{max} = 525$ nm ($\varepsilon = 72{,}000$ cm^{-1} M^{-1}).

*4,4-Difluoro-1,3,5,7-tetramethyl-6-carboxyethyl-2-[2-(4-isothiocyanatophenyl)ethylcarbamylethyl]-4-bora-3a,4a-diaza-s-indacene **(6)***

Compound **5** (10 mg, 19.5 μmol) was dissolved in chloroform. $NaHCO_3$ (350 mg, 4.2 mmol) and thiophosgene (76 μl, 1.0 mmol) were added. The reaction suspension was stirred at RT for 3 min. The reaction mixture was filtrated and evaporated to dryness. The crude product was purified with column chromatography using silica as a stationary phase and dichloromethane:acetone:acetic acid (100:16:1, v:v:v) as eluent. Fractions containing the product (**6**) were combined and evaporated to dryness. The product was dried in desiccator over silica gel to yield in 10 mg (93%) of compound **6**.

^{1}H NMR (Bruker Avance DRX 500, $CDCl_3$ (5% CD_3OD), 500 MHz, δ ppm): 7.15 and 7.07 (4H, 2d, $J = 8.6$ Hz, Ph), 6.98 (1H, s, Ar—CH═), 5.67 (1H, bs, —NH—), 3.42 (2H, m, —CH_2—), 2.72(3*2H, m, 3-CH_2—), 2.51 (3H, s, CH_3), 2.48 (3H, s, CH_3), 2.44 (2H, t, $J = 7.8$ Hz, —CH_2—), 2.23 (2H, t, $J = 7.2$ Hz, —CH_2—), 2.20 (3H, s, CH_3), 2.16 (3H, s, CH_3).

MS (MALDI): found 553 (M + H), 534 (M + H − 19), calculated 553 (M + H).

UV–Vis (MeOH): $\lambda_{max} = 525$ nm ($\varepsilon = 75{,}000$ cm^{-1} M^{-1}).

Photometry

Few milligrams of compound **3**–**6** were weighed and dissolved in DMF (200 μl). The DMF solutions were diluted either with methanol or with phosphate buffer (Na_2HPO_4 50 mM, NaCl 150 mM, NaN_3 10 mM, pH 7.4) by factor of 1000 and the UV-Vis spectra were recorded. The molar extinction coefficients were calculated from the spectral data according to the Beer's law.

Fluorescence Spectroscopy

The same stock solutions that were used for measurement of the absorption spectra were used also for measurement of fluorescence emission spectra and two-photon excited fluorescence efficiencies. The stock solutions were diluted to concentration of 125 nM with methanol. The fluorescence emission spectra were recorded by using in-house constructed spectrofluorometer which employed a halogen lamp illumination source (480 nm, ±5 nm FWHM). The fluorescence emission spectra were recorded in wavelength range of 500–650 nm. The fluorescence spectrum of each dipyrrylmethene-BF_2 dye was integrated, the integral was divided by molar absorptivity (at 480 nm) and the resulting values were compared to the value obtained for the reference compound (Rhodamine 6G in EtOH [29]). The measurement of compound **3** was performed immediately after dilution with methanol because aggregate formation was found to take place upon standing in methanol resulting in decrease in fluorescence intensity. The fluorescence intensity of compound **5** was measured also in methanol solution containing 100 mM HCl. The two-photon excited fluorometry was carried out using a microfluorometer described in detail in the literature [25]. This fluorometer employed Nd:YAG microchip laser (average power 70 mW, repetition rate 17 kHz, nominal pulse length 1 ns) as illumination source and BG39 short pass emission filter.

Labeling of Immunoglobulin G with Dipyrrylmethene-BF_2

Labeling with dipyrrylmethene-BF_2 succinimidyl ester (**4**): To the solution of 200 μg (1.25 nmol) of monoclonal mouse IgG anti-hAFP (clone 5108) in 56 μl phosphate buffered saline (Na_2HPO_4 10 mM, NaCl 150 mM, pH 7.4) 10 fold excess of labeling reagent **4** in anhydrous DMF (1.9 μl, $c = 7.5$ mM) was added. 5.6 μl of $NaHCO_3$ (1 M, aq) was added and the mixture was incubated at room temperature for 2 hr. The product was purified with NAP-5 gelfiltration column using phosphate buffered saline (50/150, 10 mM NaN_3, pH 7.4) as eluent. The fast moving orange fraction was collected and the labeling degree was determined spectrophotometrically using the following ε-values: 68,000 cm^{-1}M^{-1} at 525 nm for the label and 210,000 cm^{-1}M^{-1} at 280 nm for IgG. Labeling degree of 6 fluorophores per IgG was obtained.

Labeling with dipyrrylmethene-BF_2 isothiocyanate (**6**): To the solution of 370 μg (2.31 nmol) of monoclonal mouse IgG anti-hAFP (clone 5108) in 330 μl phosphate buffered saline (Na_2HPO_4 10 mM, NaCl 150 mM, pH 7.4) 10 fold excess of labeling reagent **6** in anhydrous DMF (4.3 μl, $c = 5.4$ mM) was added. 33 μl of Na_2CO_3 (1 M, aq, pH 9.5) was added and the mixture was incubated at room temperature for 24 hr. The product was purified with NAP-5 gelfiltration column using phosphate buffered

saline (50/150, 10 mM NaN_3, pH 7.4) as eluent. The fast moving orange fraction was collected and the labeling degree was determined spectrophotometrically. Labeling degree of 3.4 fluorophores per IgG was obtained.

Labeling of Immunoglobulin G with *R*-Phycoerythrin

To the solution of 200 μg (1.29 nmol) of monoclonal mouse IgG anti-hAFP (clone 5108) in 56 μl phosphate buffered saline (Na_2HPO_4 10 mM, NaCl 150 mM, pH 7.4) 10 fold excess of GMBS in anhydrous DMF (0.5μl, $c = 26.7$ mM) was added. 5.6 μl of $NaHCO_3$ (1 M, aq) was added and the mixture was incubated at room temperature for 1.5 hr. The product was purified with NAP-5 gelfiltration column (Amersham Pharmacia Biotech, Uppsala, Sweden) using phosphate buffered saline (50/150, 10 mM NaN_3, pH 7.4) as eluent.

To the solution of *R*-phycoerythrin-SPDP conjugate in phosphate buffered saline (Na_2HPO_4 10 mM, NaCl 150 mM, pH 7.4) (180 μl, 1.5 nmol) 2.4 μl of dithiothreitol (DTT, 124 mM in DMF) was added. The reaction mixture was incubated at room temperature for 5 hr. The product was purified by using NAP-5 gelfiltration column and by using phosphate buffered saline (50/150, 10 mM NaN_3, pH 7.4) as eluent. The last few drops of the product fraction were discarded to ensure the product being free from DTT.

The two solutions (IgG-maleimide and RPE-SH) were combined and incubated in RT for 24 hr. The RPE-IgG conjugate was purified by Superdex 200 gelfiltration column using phosphate buffered saline (Na_2HPO_4 50mM, NaCl 150 mM, pH 7.4) as eluent at flowrate of 0.8 ml/min.

Immunoassay

Mouse monoclonal IgG anti-hAFP (clone 5107) was covalently coupled to the microparticles by using a standard EDC coupling method [10]. The concentration of the stock suspension of the coated microparticles was determined in Burker cammare, and was diluted with assay buffer (TRIS-HCl 50 mM, NaCl 150 mM, 10 mM NaN_3, 0.5% bovine serum albumin, 0.01% Tween 20, pH 8.0) to concentration of 1.5 10^7 pcs/ ml. The labeled mouse monoclonal IgG anti-hAFP was diluted with assay buffer to final concentration of 8 nM. The hAFP analyte standard was diluted with assay buffer to concentrations of 0, 0.1, 1, 10, 100, 400 and 1000 ng/ml. 5 μl of microparticle suspension and 5 μl of tracer were mixed and 10 μl of analyte standards were added. The reaction mixtures were incubated for 24 hr in 20°C under continuous shaking and measured in the same reaction cuvettes. The reaction mixtures were measured with two ArcDia™ TPX-microfluorometers, one equipped with a microchip Nd:YAG laser and another equipped with a femtosecond Nd:Glass laser. The measuring time was 1 min per well.

CONCLUSIONS

Here we have described simple and efficient synthesis of novel dipyrrylmethene-BF_2 chromophore and fluorescent labeling reagents thereof. The fluorescent IgG-conjugate of the dipyrrylmethene-BF_2 labels was tested in a separation-free immunoassay of hAFP by using the separation free ArcDia™ TPX assay technique. The performance of the dipyrrylmethene-BF_2 labels as a tracer in the immunoassay were compared to that of the *R*-phycoerythrin, which is known to exhibit one of the highest two-photon absorption cross-sections and fluorescence quantum yield close to unity. The two-photon excited fluorometry was carried out with two different fluorometers. The first contained Nd:YAG microchip laser providing nanosecond pulses and 17 kHz repetition rate, and the other contained Nd:Glass laser providing 140 femtosecond pulses with repetition rate of 110 MHz. With both lasers the dipyrrylmethene-BF_2 label provided higher assay sensitivity and better signal-to-noise ratio than that of the *R*-phycoerythrin. The results show that the novel dipyrrylmethene-BF_2 labels are especially suited in applications that are based on two-photon excited fluorescence. Also, the results show that the performance of a fluorescent label in two-photon excitation applications is strongly dependent on the properties of the laser used for excitation.

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