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Dipyrrylmetheneboron Difluorides as Labels in Two-Photon Excited Fluorometry. Part I-Immunometric Assays

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Seven different two-photon excitable dipyrrylmetheneboron difluoride labels (dipyrrylmethene-BF_2 labels) and a frequently used TAMRA label were conjugated to mouse IgG against α-fetoprotein in variable substitution degrees. Altogether 40 IgG conjugates were prepared, and studied with respect to one-photon absorption and emission properties, and two-photon fluorescence efficiency using 1064 nm laser as illumination source. Performance of the IgG conjugates as tracers in a separation-free immunometric assay of α-fetoprotein was evaluated using two-photon excitation assay technology, ArcDia^{TM} TPX. The results show that the dipyrrylmethene-BF_2 labels provide subpicomolar sensitivity, which is an order of magnitude better than that of TAMRA label. The effect of chromophore structure and substitution degree of IgG-label conjugates on the assay performance is discussed.

KEY WORDS: Two-photon excited fluorescence; dipyrrylmethene-BF_2; separation-free immunoassay; ArcDia^{TM} TPX; labeling; bioaffinity assay.

INTRODUCTION

During the last decade fluorometry has become the leading analytical tool in biomedical research laboratories. Many techniques of increasing practical significance, such as fluorescence imaging, flow cytometry, real-time PCR, DNA sequencing and gene arrays, rely on fluorescence detection. The main advantages of fluorometric techniques include simplicity of assay routines and instrumentation, low cost and good availability of fluorescent probes and reagents. The applicability of conventional prompt fluorescent probes in quantitative bioassays, however, is severely hampered by Raleigh and Raman scattering and autofluorescence from biological samples and optical components, which tend to increase the background signal (=noise), thus to decrease the analytical sensitivity of the detection technique. Due to this drawback, prompt fluorescent dyes have had little practical value as labels for bioaffinity assays with sub-picomolar sensitivity.

To overcome the lack of sensitivity of the conventional fluorometric techniques, several new and innovative fluorescence detection technologies have been developed during the last two decades. Most of these technologies are based on suppression of the background signal. Such technologies include, for example (i) time-resolved fluorometry in combination with long-decaying lanthanide chelates [1,2] and phosphorescent metalloporphyrin labels [3,4], (ii) bioaffinity assay technology LabMAP^{TM} (Luminex Inc.) which employs microparticles as bioaffinity reaction carrier and fluorescence detection by flow cytometry [5], (iii) electroluminescence technology [6,7] characterized with excitation with electric potential instead of UV or visible illumination. More recently, a new detection technique, ArcDia^{TM} TPX technique (Arctic Diagnostics Oy), for bioaffinity assays was developed. This technique employs microspheres as solid carrier for bioaffinity reactions and detect fluorescence signal from individual microspheres. The technique is based *on two-photon*

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excited fluorescence detection (TPE) and enable separation free bioaffinity assays from microvolumes in subpicomolar detection limit [8–11].

In two-photon excited fluorescence (TPE), excitation and emission processes take place in essentially different wavelength ranges. As an outcome of this feature, scattering of illumination light and fluorescence from optical components can be easily eliminated by optical filtering. This gives fluorescence signal, which is practically free from scattering and background fluorescence. Another basic feature of TPE is that the probability of excitation is dependent on the second power of illumination intensity. This property leads to fluorescence excitation in sharply restricted 3-dimensional focal point, while only minimal fluorescence outside of this volume is generated [12–15]. It has been shown lately that optimal fluorescent labels for TPE (and for the ArcDia TPX) do not share the common photophysical properties with labels good in one-photon excited fluorescence [16,17]. Instead, fluorescent labels with prompt fluorescence decay, with short Stokes' shift and moderate excitation cross section has turned out to provide the highest performance for bioaffinity assay in the ArcDia TPX assay platform. From the group of prompt fluorescent dyes, including derivatives of xanthenes, cyanines, and phycobiliproteins, *dipyrrylmethene-BF_2 dyes* has been found most potential for TPE, in particular for the ArcDia TPX assay technique. On the basis of this group of compounds, we developed a series of hydrophilic labeling reagents with variable excitation and emission maxima. The synthesis and fluorescence properties of these compounds under two-photon excitation was recently reported [16,17]. Here we describe the use of the same dipyrrylmethene-BF_2 compounds as labels in immunoassay. We report a study of conjugation performance of the dipyrrylmethene-BF_2 labeling reagents, a study of photophysical properties of label-antibody conjugates, and a study of the performance of the antibody conjugates as tracers for immunometric assay of a clinically relevant analyte, α-fetoprotein, using the ArcDia TPX assay technique.

EXPERIMENTAL

Reagents

The reagents were purchased either from Sigma or Fluka and used without further purification. *N,N*-dimethylformamide (DMF) was p.a. grade from Lab-Scan and was dried over molecular sieves (4 Å). Water was Millipore™ Rios3 grade. 6-Carboxytetramethylrhodamine succinimidyl ester (6-TAMRA-SE) was purchased from Molecular Probes (Eugene, OR, USA). Monoclonal mouse IgGs against human α-fetoprotein (hAFP, clones 5108 and 5107) were purchased from Medix Biochemica (Kauniainen, Finland) and the hAFP standard (X0900) was from Dako AS (Denmark). Microspheres (3.2 μm in diameter, carboxy modified microspheres, PC05N, COOH-group/0.852 nm^2) were purchased from Bangs laboratories (Fishers, IN, USA). Dipyrrylmethene-BF_2 labeling reagents (BF-labels) were synthesised according to previously published methods [16,17]. NAP-5 and Superdex-200 gelfiltration columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Microtitration plates (384-well plate, TC-grade with black walls and clear bottom) were obtained from Greiner (Frickenhausen, Germany).

Instrumentation

The UV-Vis spectra were recorded on a SD-2000 Ocean Optics single beam fiber optic diode array spectrophotometer. The fluorescence emission spectra were recorded by using in-house constructed spectrofluorometer which employed argon–krypton laser as illumination source (laser line at 514 nm or 531 nm was used for excitation).

Two-photon excitation microfluorometry was performed with ArcDia TPX Platereader (Arctic Diagnostics Oy, Turku, Finland), a microfluorometer specially designed for reading of two-photon excited fluorescence from the surface of individual polymer microspheres. The instrument was recently described in detail [10,18]. The instrument is equipped with a passively Q-switched, diode pumped, micro-chip Nd:YAG laser (1064 nm, average power 70 mW, repetition rate 17 kHz, nominal pulse length 1 ns), and an emission filter that enables signal collection in the range of 530–610 nm.

General Procedure for Labeling of IgG

A small amount of labeling reagent was weighed and dissolved in anhydrous DMF to give label stock solution. An aliquot of this solution was diluted with methanol by factor of 1000 and UV-Vis spectrum was recorded. Concentration of the DMF stock solutions were calculated using the molar extinction coefficients listed in Table I. The stock solution was diluted with DMF to get a working solution of 5 mM. A variable volume of this solution (corresponding to 3, 6, 10, 15 or 20 fold molar excess) was added 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 10mM, NaCl 150 mM, pH 7.4). The volume ratio of DMF in the labeling reactions remained below 15% of total reaction volume. 5.6 μl

Table I. Absorption and Fluorescence Data of the Labeling Reagents

Dye	λ_{Abs} (0.1% TX-100) [nm][a]	λ_{em} (0.1% TX-100) [nm][a]	ε (MeOH) [cm^{-1} M^{-1}][a]	ε (0.1% TX-100) [cm^{-1} M^{-1}][a]	ε (PBS) [cm^{-1} M^{-1}]	CF (A_{280}/A_{max}, PBS) [a.u.]
BF523	524	533	75000	75000	68000	0
BF530	533	557	64000	54000	48000	0.44
BF545	547	574	66000	64000	43000	0.30
BF560	565	579	82000	69000	68000	0.15
BF568	574	574	132000	118000	73000	0.27
BF580	584	598	122000	124000	69000	0.28
BF585	589	603	83000	74000	62000	0.27
TAMRA	547 (MeOH)[b]	573 (MeOH)[b]	91000[b]	—	65000[b]	0.30[b]

[a] Values are obtained from [16,17].
[b] Values are obtained from [19].

of $NaHCO_3$ (1 M, aq) was added and the mixture was incubated at room temperature for 3 h. 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 coloured fraction was collected. The labeling degree of the IgG conjugates was determined spectrophotometrically using the extinction coefficients (PBS) and correction factors (CF) given in Table I. Molar absorptivity of 210,000 $cm^{-1}M^{-1}$ at 280 nm was used for IgG.

Fluorescence Spectroscopy

The same DMF stock solutions that were used for labeling 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 water containing 0.1% Triton X-100, and the label IgG conjugates were diluted to the same concentration with phosphate buffered saline (Na_2HPO_4 50 mM, NaCl 150 mM, 10 mM NaN_3, pH 7.4). The fluorescence emission spectra were recorded in wavelength range of 525–700 nm. The excitation wavelength was 514 nm for BF523 and BF530 labels, and 531 nm for TAMRA, BF545, BF560, BF568, BF580 and BF585 labels. Two–photon excitation fluorometry was carried out with the ArcDia TPX platereader. The same label and conjugate solutions were dispensed into wells of a 384-well plate and measured with the ArcDia TPX platereader using liquid measurement mode and 10 s integration time.

Immunoassay

Mouse monoclonal IgG anti-hAFP (clone 5107) was covalently coupled to microspheres by using a standard EDC coupling method [11]. Concentration of stock suspension of coated microspheres was determined with Multisizer™ 3 Coulter Counter (Beckman Coulter Inc.), followed by dilution 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. Labeled anti-hAFP IgGs (tracers) were diluted with assay buffer to concentration of 8 nM. AFP analyte standard was diluted with assay buffer to concentrations of 0, 0.1, 1, 10, 100, 400 and 1000 ng/ml. 5 μl of microsphere suspension and 5 μl of tracer were dispensed into a well of microtitration plate followed by addition of hAFP standards (10 μl). The reaction mixtures were incubated for 24 hr at 20°C under continuous shaking and measured with ArcDia TPX platereader using the particle measurement mode. In particle measurement mode the microspheres are continuously tracked by the three dimensional scanner. When a microsphere appears in the focus, the confocally arranged scattering detector detects the back-scattered illumination light from microsphere, and the scanning is stopped. The two-photon excited fluorescence is measured in coincidence with the back-scattered illumination light. Once the scattering signal vanishes the scanner starts again to find a new microsphere. The measuring time was 60 s per well. In order to improve the precision and accuracy of results, the individual datapoints (microspheres) characterized with the optical trap duration time less that 5 ms were omitted [18]. The bioaffinity signal was taken as a mean value of the fluorescence signal (intensity) of the remaining individual microspheres.

RESULTS AND DISCUSSION

Labeling of IgG

Monoclonal IgG antibody against hAFP was labeled with seven different dipyrrylmethene-BF_2 labels (=BF-labels) and with TAMRA. TAMRA was chosen as a reference compound since in preliminary studies this label

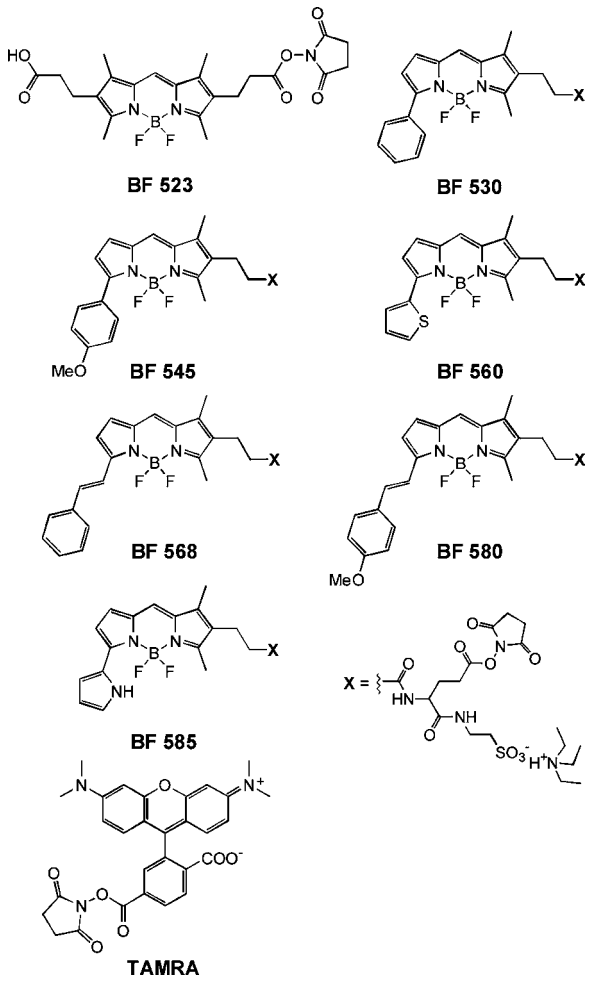

Fig. 1. Chemical structures of the labeling reagents.

provided good assay performance in comparison to other commercial labels, including ATTO™ (Atto-Tec GmbH), Alexa™ (Molecular Probes Inc.) and Cy™ (Amersham Biosciences ltd.) dyes (unpublished results). In addition TAMRA is well characterised and widely used in the field of bioanalytics. The chemical structures of the labeling reagents are shown in Fig. 1. The labeling reagents were all in form of succinimidyl ester, which is reactive towards terminal amino groups and lysine residues of proteins and peptides. The labeling was performed in slightly alkaline medium (pH 8.3) with IgG concentration of 2 mg/ml and incubation of 3 hr. The molar excess of the labeling reagents were varied providing IgG conjugates with variable substitution (labeling) degrees. Substitution degrees were determined spectrophotometrically using the extinction coefficients (PBS) and correction factors (CF) given in Table I. The substitution degrees of the IgG conjugates are presented in Fig. 2 as a function of molar excess of the labeling reagent. BF523 label was found to provide highest substitution degrees, from 2 to 10 labels per IgG, while BF560 and TAMRA provided the lowest substitution degrees, from 1 to 3 labels per IgG.

All the BF labels showed small changes in their absorption spectrum upon conjugation to IgG. The molar absorptivity seems to slightly decrease upon conjugation while the integral of the main absorption peak remain constant (data not shown). In other words, conjugation reaction is associated with broadening of the absorption bands. With all BF labels a slight increase in broadening of the absorption band was observed with increasing substitution degree. For example, the unconjugated BF530 label show FWHM (full width of half maximum) of 53 nm whereas the IgG conjugate with a substitution degree of 2.4 shows FWHM of 64 nm, and with substitution degree of 5.7 FWHM is 69 nm. In all cases the broadening was greater at the blue edge than at the red edge of the absorption peak. When compared to the free labels, also a small red shift (1–2 nm) in the position of the absorption maximum was observed upon conjugation to IgG. The shift was found not to depend on the substitution degree. These conjugation related changes in absorption spectra tend cause error in determination of substitution degree if not appropriately corrected. This is the case especially with the TAMRA label, which exhibits remarkable change in absorption spectrum upon conjugation [19,20]. This phenomenon has been accounted to originate from dye aggregates or from hydrophobic interactions between the protein and TAMRA [19].

Fluorescence Spectroscopy

Fluorescence emission spectra of the free labels and the corresponding IgG conjugates were recorded using argon–krypton laser for illumination. Fluorescence emission maximum of all labels and IgG conjugates located between 530 and 610 nm. With all BF labels conjugation to IgG caused a slight broadening, and a small (1 to 2 nm) red shift of emission peak. The shifts in emission maxima correlated well with the shifts observed in the absorption spectra. In contrast to absorption measurements, the red shift in fluorescence spectra increased slightly with increase in substitution degree. This phenomenon can be encountered as a result of energy transfer between the labels in same IgG molecule.

Quantitative two-photon excited fluorometry was performed using the ArcDia TPX-platereader. The free labels were measured in equimolar water solutions containing 0.1% Triton X-100 (TX-100) and the corresponding IgG conjugates in phosphate buffered saline. The data

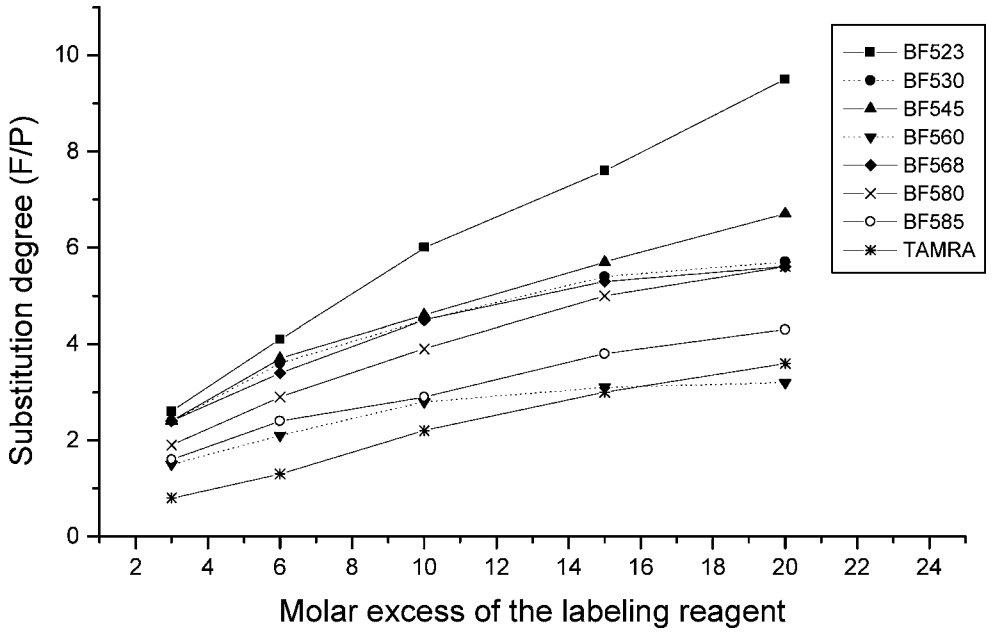

Fig. 2. Effect of excess of labeling reagent on substitution degree of IgG conjugates.

are summarised in Table II. For the free labels, the highest TPE signal was obtained with the BF560 label (250 AU) whereas the lowest signal was obtained with the BF585 label (20 AU). This result is in agreement with our previous study [17]. Two-photon excited fluorescence of the IgG conjugates was measured in equimolar concentrations in respect to IgG. The result is presented as a function of substitution degree in Fig. 3. With all labels a clear bending of curve and finally a negative slope of the curve can be observed with increased substitution degree. With low-substitution degrees TPE signal of the IgG conjugates of different labels correlate quite well to the results obtained in measurements of the free labels. At low-substitution degrees, the highest TPE signal is obtained with BF560 conjugates, and the signal of BF 580 and BF585 conjugates are clearly the lowest. At higher substitution degrees, however, signal from BF545 label exceeds the signal of BF560. This indicates that BF545 suffers less from self-quenching than BF560 label. In Fig. 4. TPE efficiency per label is presented as a function of substitution degree. The figure shows decreasing curves for all labels. This means that fluorescence efficiency from individual labels decreases as the substitution degree increases. This kind of decrease (self-quenching) is common phenomenon with fluorescence labels. It can be accounted as consequence of emission—reabsorption process, also called "the inner filter effect," or due to dynamic interaction between label molecules. The curve of the BF560 label is clearly different than the others, decreasing more rapidly and thus indicates, that the BF560 label is most prone to the self-quenching process.

The probability of self-quenching of a fluorescent label is generally considered to be proportional to the overlap integral of excitation and emission spectra. The difference in the slopes between the curves of the two most intense labels, BF560 and BF545, could easily be explained by different stokes shifts and different overlap integrals (data not shown) of absorption and emission bands. However, when looking at the results of other labels in detail, it can be found that there are no relationship between stokes shifts and self-quenching. The BF568 label with the shortest stokes shift, only 8 nm, shows equal self-quenching as the BF530 label, which has Stokes shift of 24 nm. Also, a clear difference can be observed in the shape of the curves between labels with equal stokes shifts, BF560 and BF585 (or BF580). This suggests that the mechanism of self-quenching is not based on emission re-absorption mechanism but rather on non-radiative energy transfer between the label molecules.

Compared to the measurements of the free labels, all IgG-label conjugates showed remarkably (>50%) decreased fluorescence intensities (See Table II). This conjugation-related quenching seems to be strongest with the phenyl-substituted (BF530 and BF545) labels and with the phenylethenyl-substituted (BF568 and BF580) labels. Fluorescence efficiency of the alkyl-substituted BF523 label, heteroaryl-substituted BF585 and BF560 labels and TAMRA are also suffering from self-quenching but not as

Table II. Fluorescence Efficiencies of the Label-IgG Conjugates, Immunoassay Data and Signal-to-Background Ratios

	Solution measurements			Immunoassay data		
Label	*F*/*P*	TPE per IgG (a.u.)[a]	TPE per label[b]	B kg (0 ng/ mlAFP)	Signal (50 ng/ml)	*S*/*B*-ratio (50 ng/ml)
BF523	Free	65				
	2.6	64	38%	4.51	73.6	17.3
	4.1	75	28%	4.24	85.1	20.1
	6	**64**	**16%**	**3.49**	**72.0**	**20.6**
	7.6	53	11%	3.25	66.1	20.3
	9.5	35	6%	3.46	59.1	17.1
BF530	Free	182				
	2.4	53	12%	4.14	107.7	26.0
	3.6	60	9%	4.96	128.0	25.8
	4.5	**68**	**8%**	**5.32**	**158.6**	**29.8**
	5.4	65	7%	5.75	164.8	28.7
	5.7	69	7%	5.92	173.7	29.3
BF545	Free	207				
	2.4	94	19%	3.72	49.2	13.2
	3.7	122	16%	3.82	46.9	12.3
	4.6	**136**	**14%**	**3.90**	**66.9**	**17.2**
	5.7	134	11%	5.99	83.1	13.9
	6.7	132	10%	5.19	82.1	15.8
BF560	Free	250				
	1.5	98	26%	4.27	76.4	17.9
	2.1	116	22%	4.61	114.7	24.9
	2.8	**131**	**19%**	**4.76**	**134.5**	**28.3**
	3.1	130	17%	6.21	141.0	22.7
	3.2	122	15%	6.04	129.7	21.5
BF568	Free	188				
	2.4	53	12%	2.85	29.8	10.4
	3.4	60	9%	2.52	30.9	12.3
	4.5	**68**	**8%**	**3.03**	**37.9**	**12.5**
	5.3	65	7%	3.99	46.7	11.7
	5.6	69	7%	3.53	51.2	14.5
BF580	Free	58				
	1.9	**12**	**11%**	**0.6**	**5.0**	**8.4**
	2.9	16	10%	0.74	5.6	7.6
	3.9	17	8%	0.98	8.2	8.3
	5	17	6%	1.12	8.5	7.6
	5.6	18	6%	1.29	9.1	7.1
BF585	Free	20				
	1.6	11	34%	0.76	4.5	5.9
	2.4	19	40%	1.11	6.4	5.8
	2.9	17	29%	1.82	8.8	4.8
	3.8	**19**	**25%**	**1.87**	**19.1**	**10.2**
	4.3	18	21%	1.85	12.1	6.5
TAMRA	Free	50				
	0.8	19	48%	2.54	11.6	4.5
	1.3	25	38%	2.43	15.9	6.5
	2.2	**32**	**29%**	**2.72**	**19.4**	**7.1**
	3	33	22%	3.10	21.3	6.9
	3.6	31	17%	3.25	22.5	6.9

[a] Two-photon excited fluorescence yields per IgG in arbitrary units.
[b] Two-photon excited fluorescence yields per label relative to corresponding unconjugated label [%].

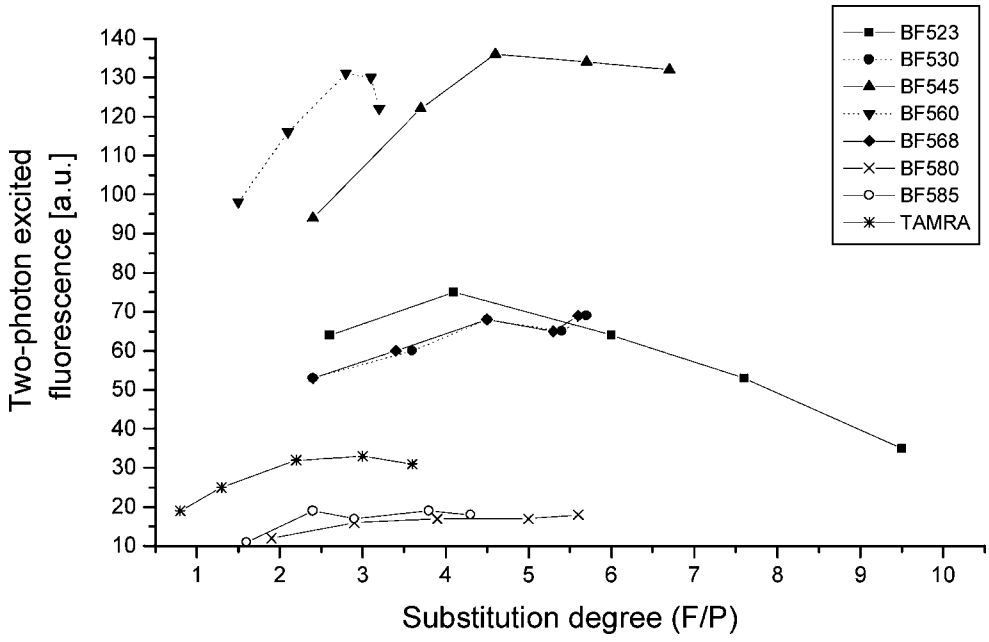

Fig. 3. Fluorescence efficiency of IgG conjugates with variable substitution degrees.

strongly as the other labels. Fluorescence of the IgG conjugates was measured also in the presence of a detergent, Tween 20 in 0.01% concentration (data not shown). As a result, the fluorescence intensity of BF-IgG conjugates increased 30%, on an average. In our previous study with the free BF-labels [17], addition of a detergent was found to increase both molar absorptivity and fluorescence quantum yield. The increase was accounted to originate from ability of detergent to prevent formation of dye dimers or aggregates. In case of IgG-conjugates, theory of aggregate

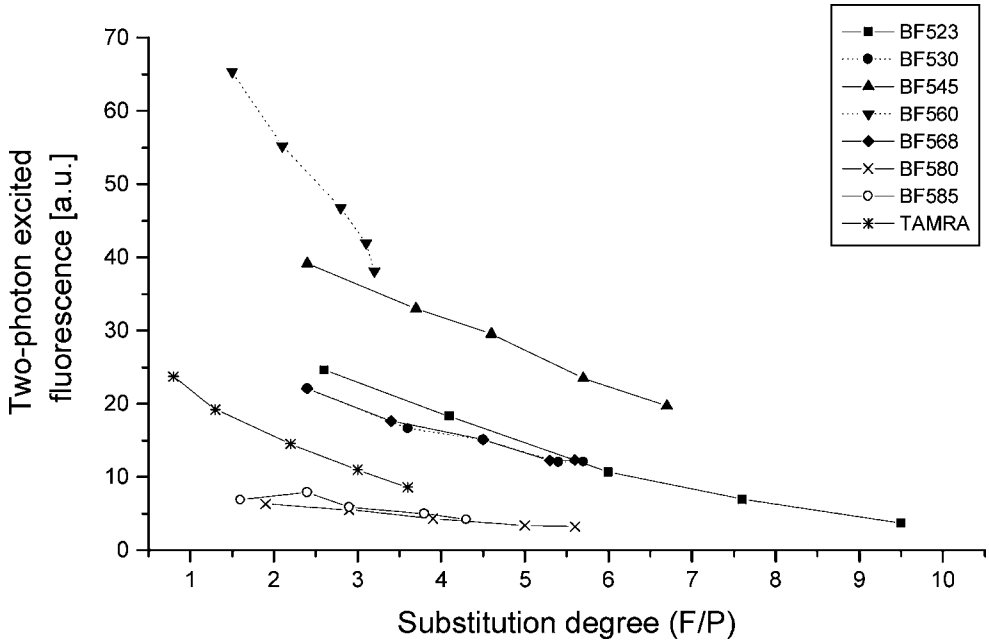

Fig. 4. Two-photon excited fluorescence per label as function of substitution degree.

formation does not seem likely, but rather, hydrophobic interactions between the protein and the label appear more reasonable explanation for the quenching phenomenon.

Immunoassay

Assay Principle

Performance of the IgG-label conjugates was tested in a separation-free immunometric assay using the ArcDia TPX assay technique. The separation free assay format is based on the use of microspheres as solid reaction carriers and the non-linear character of two-photon excitation, which results in generation of fluorescence only in diffraction limited focal volume of the laser illumination. In immunometric assay, bioaffinity complexes, consisting of a catching antibody, the analyte and a fluorescent tracer antibody, are formed on the surface of individual microspheres. When such microsphere is brought into focus by optical forces of the illuminating laser, a fluorescence burst is generated and the intensity of the burst is proportional to the number immunocomplexes on the surface of microsphere. The optical configuration of the fluorometer and the physical phenomena related to the measurement process has been described in detail in our previous publications [10,18].

In assays carried out with the ArcDia TPX technique, the major source of signal noise (background signal) originates from the unbound fraction of the fluorescent tracer. Therefore, the concentration of the tracer is critical for the performance of the assay, and consequently, the tracer concentration is a subject of careful optimisation. Another parameter affecting to the sensitivity and dynamic range of the assay is the amount of microspheres in the reaction mixture. In this study both the amount of microspheres and the concentration of the tracer were kept constant to allow comparison between the different tracers.

Assay Conditions

The model assay was an immunometric assay of human α-fetoprotein (hAFP). The microspheres were coated with a specific antibody against hAFP. Labeled monoclonal mouse IgGs against another epitope of hAFP were used as a tracer. Concentration of tracer was in all measurements 4 mM and the amount of microspheres was 75,000 pieces per assay. The reaction mixtures were incubated for 24 hr prior to measurements to ensure equilibrium between bound and free fraction. After incubation, two-photon excited fluorescence from the surface of individual microspheres is measured separation free, directly from the reaction mixture using the ArcDia TPX platereader. In this study, fluorescence emission from each assay replicate was integrated for 60 s. During this measurement time, typically 50 to 70 individual microspheres were found and measured. In order to increase assay precision, the fluorescence data were filtered by excluding exceptionally short optical events. This filtering process removes most events originating from foreign particles, such as dust and possible reagent aggregates.

Conjugate Performance

The immunoassay data at analyte concentration of 0 and 50 ng/ml are presented in Table II. These datapoints were used for calculation of signal-to-background ratios, which is considered in this study as the main figure of merit of the assay performance. Analyte concentration of 50 ng/ml was chosen, since at this concentration all labels give reasonable signal levels, and on the other hand, this concentration is clearly below the maximum binding capacity of the microspheres. According to the results, the best signal-to-background ratios are obtained with the BF530 and BF560 conjugates, whereas the poorest ratios were obtained with the BF580, BF585 and TAMRA conjugates (see Table II). Conjugates providing the best signal-to-background ratio within each label-IgG series are marked bold, and the assay curves of these particular conjugates are presented in Fig. 5. Each data point represents mean of two assay replicates. In terms of signal-to-background ratio and absolute signal levels, the BF530 and BF560 labels provide clearly the best assay performance. A surprise was the low-signal level that were obtained with the BF545 conjugates. Compared to the BF530 conjugates, the BF545 conjugates gave in solution measurement mode two times more signal than BF530 conjugates. In assay conditions, however, the ratio was opposite. The BF530 conjugates gave nearly twice the signal that was obtained with BF545 conjugates. It seems that fluorescence of the BF545 label is quenched when bound on the surface of microspheres. Similar behaviour has been observed with R-phycoerythrin (RPE) conjugates. [16]. The origin of this quenching remained unclear. Possible explanations for this phenomenon include, photophysical saturation of the dye molecules, photobleaching, excited state absorption and stimulated emission. In case of the BF545 conjugates, photophysical saturation of the dye molecules seems a most improbable explanation, since in solution measurement mode the BF545 and BF560 conjugates gave signals of the same level, whereas in assay conditions the signal of the BF545 conjugate was only half that of the BF560 tracer. In case of photophysical

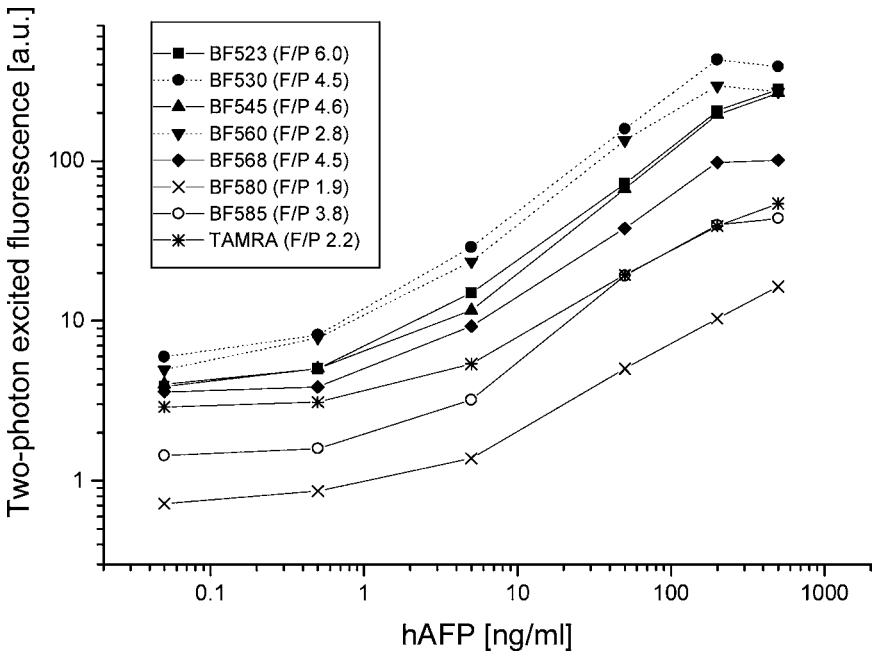

Fig. 5. Standard curves for hAFP assays using tracers with optimised substitution degrees.

saturation, the signal level is determined by quantum efficiency of a fluorophore. In free form, the quantum efficiencies for the BF545 and BF560 labels are 0.78 and 0.65 [17]. According to this study, the quantum efficiencies of the IgG conjugates are decreased when compared to the free labels. However, the ratio between the two labels remained same. This suggests that, saturation alone is not responsible for the low-signal levels obtained with the BF545 tracers in assay conditions. We are currently under way of studying photostability of BF labels under both one- and two-photon excitation and we expect these measurements to further elucidate the observed behaviour of the BF545 label. According to our preliminary results with conventional one-photon excitation, the BF545 label shows equal photobleaching rate to the BF560 and BF530 labels. However, it has been reported earlier, that with certain fluorophores the photobleaching rates under two-photon excitation can be considerably different to those of one-photon [21].

In assay conditions, the tracers with high-substitution degree gave, as expected, higher signal than the tracers with low-substitution degrees. However, the average slopes of the assay curves obtained with the conjugates of high-substitution degree are smaller than the slopes of the curves obtained with the conjugates of low-substitution degree. As an example, assay curves of the BF545 tracers with three different substitution degrees are presented in Fig. 6. When assuming Poisson distribution in label conjugation this observation can be explained. For example, according to Poison statistics the conjugate with average substitution degree of 2.4 labels per IgG, the amount of unlabeled IgG is 9%. It is likely that this portion of the conjugate molecules has the highest affinity to the analyte, thus it binds the analyte stronger than the labeled IgG molecules. The unlabeled IgG may also have a kinetic advantage over the labeled IgGs on binding to the analyte. The kinetic factors, however, do not contribute in the results of the present study because the assays were incubated for 24 hr, which has been shown to correspond to state of equilibrium [22]. At the substitution degree of 6.7 labels per IgG the amount of unlabeled IgG is only 0.1%. With this tracer, very few IgG molecules are unlabeled, and therefore, this conjugate give better signal response at low-analyte concentrations than the conjugate with average substitution degree of 2.4 labels per IgG. However, as the substitution degree increases, also the portion of inactivated (low affinity) IgG molecules increases. This results in decrease of the assay response at the high-analyte concentrations, and decrease of the average slope of the assay curve (see on Fig. 6).

Assay Precision and Lowest Limit of Detection

Assay performance of three different label conjugates was studied in more detail. The conjugates were: BF530, BF560 and TAMRA with substitution degrees of 4.5, 2.8 and 2.2 label per IgG, respectively. The assay of hAFP was repeated with these three conjugates using the

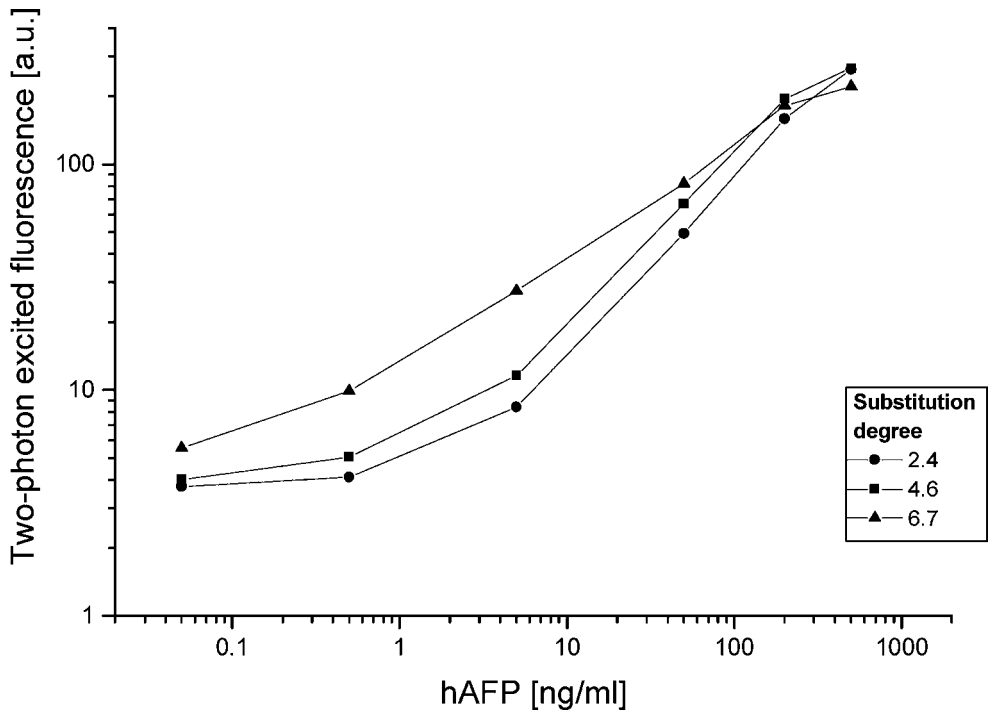

Fig. 6. Standard curves for hAFP assay using BF545 tracer with three different substitution degrees.

same assay parameters as before but this time each assay was performed with six replicates, and the negative control in 10 replicates. The results are presented in Fig. 7. The figure shows assay response curves, and the 3SD levels of the negative control. According to the figure, the BF530 conjugate provides the highest signal response and the lowest detection limit of the three conjugates. All three conjugates show flat CV profile throughout the assay range (data not shown). The average assay CVs were 5% with BF530 conjugate, 6% with BF560 conjugate and 8% with TAMRA conjugate. The lowest limit of detection was determined as an intercept of the assay curve and the 3SD (standard deviation) level of blank sample signal. The lowest limit of detection of the assay with the BF530 tracer is better by a factor of 10 and 15 when compared with BF560 and TAMRA labels. The lowest limit of detection of hAFP using the BF530 tracer was 0.05 ng/ml, which corresponds to 0.67 pM concentration. It is worth noting is that this sub-picomolar sensitivity was obtained without optimisation of assay conditions.

CONCLUSIONS

In this paper, the use of dipyrrylmethene-BF_2 fluorophores as labels for immunometric assay of human α-fetoprotein has been studied. The results show that dipyrrylmethene-BF_2 labeling reagents with succinimidyl reactive group show high reactivity in coupling reactions with immunoglobulin G proteins. Under typical labeling conditions, 2 to 6 labels per IgG molecule is incorporated. The conjugated labels show minor changes in absorption and emission spectra when compared to those of unconjugated labeling reagents. The fluorescence quantum efficiency of the labels, however, is markedly decreased on conjugation to proteins. This quenching of fluorescence was shown to follow two different types of mechanisms. The first originates from interaction of a label molecule with the protein (=conjugation related quenching) whereas the other results from interaction between two or several conjugated label residues (=self-quenching). Conjugation-related quenching was found to correlate with hydrophobicity and bulkiness of the label, and to decrease on addition of detergent. These findings suggest that further increase in hydrophilicity of the fluorophores would provide decrease in quenching, thus, increase in fluorescence efficiency of conjugated labels.

All IgG conjugates were tested as tracers for a separation free immunometric assay using the ArcDia TPX assay technique. According to the results, dipyrrylmethene-BF_2 fluorophores perform superior to TAMRA label. Of the seven dipyrrylmethene-BF_2 labels included in this study, phenyl substituted BF530 label provided the best performance in immunometric assay of α-fetoprotein. With this label the lowest limit of detection for α-fetoprotein was

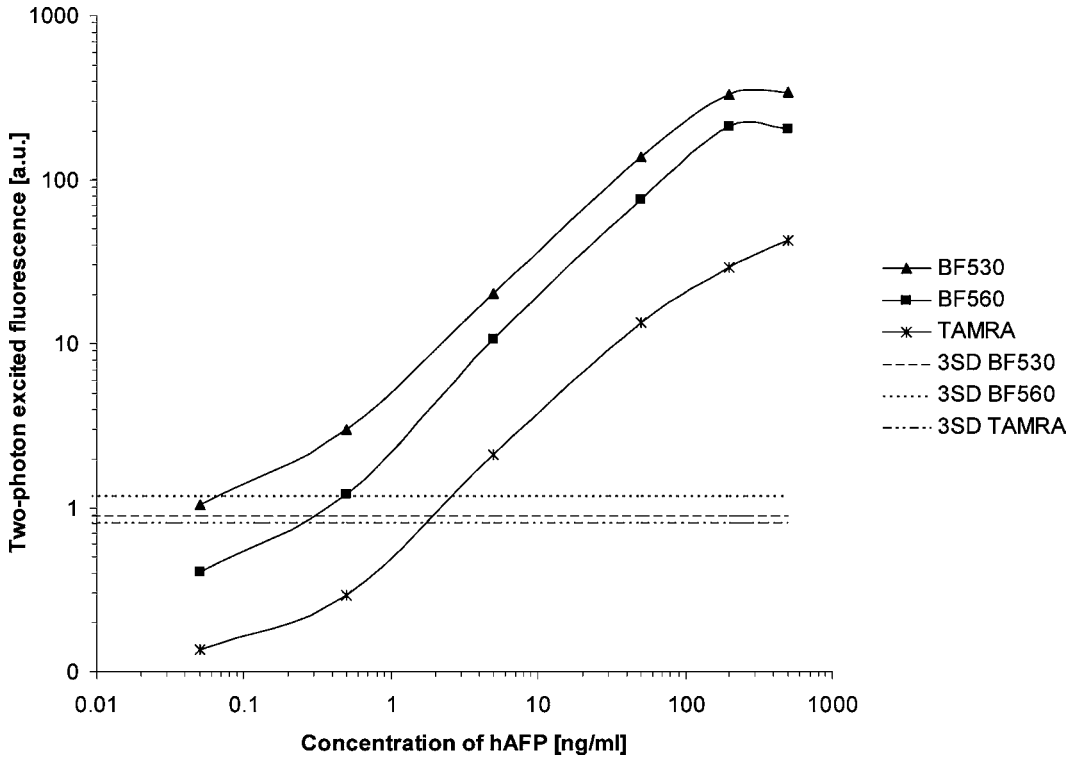

Fig. 7. Background subtracted standard curves for hAFP assays using BF530, BF560 and TAMRA tracers, and 3SD levels of negative control samples.

670 fM. This detection limit is considered excellent for a separation free assay method, and it exceeds clearly the clinical reference range for α-fetoprotein [23]. Furthermore, the results show that the performance of the label conjugates in assay conditions do not correlate with two-photon excited fluorescence yields of the same conjugates in homogeneous solution. This finding suggests that different excitation–relaxation mechanisms dominate for the labels bound on microsphere surface and for the labels in liquid phase.

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