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Dipyrrylmetheneboron Difluorides as Labels in Two-Photon Excited Fluorometry. Part II–Nucleic Acid Hybridization Assays

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Five two-photon excitable dipyrrylmetheneboron difluoride labels (dipyrrylmethene-BF_2 labels) with fluorescence emission maximum between 530 and 590 nm, and a frequently used rhodamine label, TAMRA, were conjugated to aminomodified oligonucleotides. The performance of the labeled oligonucleotides was studied in a separation-free nucleic acid hybridization assay using ArcDia^{TM} TPX bioaffinity assay technology. The results show that oligonucleotide conjugates of dipyrrylmethene-BF_2 labels provide higher two-photon excited fluorescence yield and better assay sensitivity than corresponding TAMRA conjugate. The effect of conjugation on photophysical properties of the labels and performance of the labeled oligonucleotides in separation-free hybridization assay is discussed.

KEY WORDS: Dipyrrylmetheneboron difluoride; labeling reagent; succinimidyl ester; oligonucleotide; hybridization assay; two-photon excitation fluorescence; bioaffinity assay; ArcDia TPX.

INTRODUCTION

The significance of nucleic acid hybridization as a basic tool for bioanalysis has increased remarkably during the last two decades. Analytical techniques such as DNA sequencing [1,2], real-time PCR [3], fluorescence in situ hybridization (FISH) [4,5] and gene arrays [6,7] have become routine tools in biomedical research. Most of these techniques rely on detection of prompt fluorescence which enables fast, simple and relatively low-cost assays with high throughput. The fluorescent reporter molecules that are most commonly employed in nucleic acid hybridization assays are conventional fluorescent labels or intercalating dyes, such as fluoresceins, rhodamines and cyanine dyes or ethidium bromide and SYBR Green^{TM} dyes [3,8]. Most of the commercial methods for real-time PCR monitoring, including Taqman [9,10] and Scorpion probes [11], and Molecular Beacons [12,13], are also based on prompt fluorescent label compounds and fluorescence energy transfer (FRET) principle.

Besides conventional prompt fluorescent dyes, other type of photoluminescent reporters also have been introduced for DNA assays. These reporters include long decay time molecular labels, such as lanthanide chelates [14,15] and phosphorescent metalloporphyrins [16,17], and particulate photoluminescent reporters, such as quantum dots [18,19], and inorganic phosphors [20]. The use of these alternative label technologies as research tools in DNA assays, however, have so far been very limited in comparison to the conventional prompt fluorescent dyes.

Two-photon excited fluorescence (TPE) is a technique with increasing practical value. This technique was first applied in microscopy imaging [21–23], and more recently, also in the field of quantitative bioaffinity assays [24–26]. Based on TPE and the use of polymer microspheres as solid reaction support, we recently developed a new bioaffinity assay platform, known as ArcDia^{TM} TPX technique [26–28]. This assay platform enables

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separation-free bioaffinity assays to be carried out in microvolumes with high sensitivity. The technique has been applied for several different binding assay schemes, including immunometric assays [27,29], competitive binding assays [30], assays of cell surface antigens [31], and assays for detection of single nucleotide polymorphism [28] using the minisequencing reaction principle [32].

Very recently, we designed and synthesized a series of hydrophilic fluorescent labeling reagents for two-photon excited fluorometry [33,34]. In the first part of this study [35] (Part I, see *ibid*), we investigated the performance of the new labels as tracers for immunometric assay of a protein antigen, human α-fetoprotein (hAFP). Here, in the second part of the study, we investigate the performance of the same labels in context to nucleic acid hybridization assays. We report a study of conjugation of the labels to oligonucleotides, a study of photophysical properties of the label-oligonucleotide conjugates, and finally a study of the performance of the oligonucleotide conjugates as tracers in DNA hybridization assay for a single stranded synthetic DNA target using the ArcDia TPX assay technique.

EXPERIMENTAL

Reagents

The reagents were purchased either from Pierce Chemical Company or Sigma and used without further purification. The solvents were p.a. grade and purchased either from Riedel de Haen, Fluka or Merck and used as received. Water was Millipore™ Rios3 grade. Oligodeoxyribonucleotide **1** (5′-TGA ACC AGA GGA GTT CTT GAC GCG CAA GTT GAC CCT CA-3′) and **2** (5′-Amino- C6-TGA GGG TGA ACT TGC GC-3′) were obtained from MWG AG BIOTECH (Ebersberg, Germany) and oligodeoxyribonucleotide **3** (5′-GTC AAG AAC TCC TCT GGT TCA-Spacer-C18-Amino- C6-3′) and **4** (5′-TAMRA-TGA ACC AGA GGA GTT CTT GAC-3′ were from Eurogentec (Herstal, Belgium). 6-carboxytetramethylrhodamine-succinimidyl ester (TAMRA-SE) was purchased from Molecular Probes (Eugene, OR, USA). Dipyrrylmethene-BF_2 labeling reagents (BF-labels) were synthesized according to previously published methods [33,34]. Microspheres (3.2 μm in diameter, carboxy modified microspheres, PC05N, COOH-group/0,852 nm^2) were purchased from Bangs laboratories (Fishers, IN, USA).

Labeling of Oligodeoxyribonucleotide

To a solution of 100 μg (19.2 nmol) of oligonucleotide **2** in 10 μl of water, 75 μl of sodium tetraborate buffer (100 mM, pH 8.5) was added (Tube: Nostick 0,5 ml, Alpha laboratories, UK). A solution of a labeling reagent in anhydrous DMF (15 μl, 26 mM) was added under vortex. The labeling reagent was used in 20 fold molar excess in respect to the oligonucleotide. During the first 15 min of incubation the vial was sonicated in bath (Finnsonic m03, Lahti, Finland) twice for 20 s. The reaction mixture was then incubated overnight under continuous shaking at 22°C in the dark (1100 rpm, Thermomixer Comfort, Eppendorf, Hamburg, Germany). The oligonucleotide conjugate was separated by sequential (3×) precipitation from ethanol as follows:10 μl of 3 M NaCl (aq) and 250 μl of cold absolute ethanol were added. The vial was vortexed and stored at −20°C for 30 min. The vial was centrifuged for 30 min (15000 × g) and the supernatant was discarded. The pellet was rinsed with cold ethanol (70%, 100 μl) and redissolved in water (100 μl), sonicated twice for 20 s and centrifuged for 15 min. The supernatant was transferred to a clean tube while the pellet containing unreacted dye was discarded. The precipitation procedure was repeated for additional two times. After the last precipitation, the pellet was dissolved in TEAA buffer (21 μl, TEAA 50 mM, NaN_3 10 mM). The labeled oligonucleotide was further purified by reversed phase HPLC as follows: Oligonucleotide solution in TEAA buffer (20 μl) was injected in the column (LiChroCART 125 × 3, purosphere RP-18e, loop volume 20 μl). The column was eluted using a linearly increasing gradient of MeCN in TEAA buffer. The proportion of MeCN was increased from 12% to 50% (v/v) in 20 min using a flow rate of 0.8 ml/min. Fractions of 400 μl were collected. The fractions containing pure conjugate were identified spectrophotometrically, pooled and evaporated to dryness. The dry pellet was dissolved in water (150 μl), and the purity of the pool was verified by analytical chromatography as follows: An aliquot from the preparative pool, was diluted by a factor of 2 with a solution being prepared of MeCN (1 volume equivalent) and TEAA buffer (4 volume equivalents, TEAA 100 mM, NaN_3 20 mM). This solution (25 μl) was injected, and the column was eluted with a linearly increasing gradient of MeCN (from 10% to 45%, v/v) in TEAA buffer (50 mM, NaN_3 10 mM) during 20 min at a flow rate of 0.8 ml/min. The purified oligonucleotide conjugate was analysed by spectrophotometry (SD-2000 Ocean Optics single beam fiber optic diode array spectrophotometer) and by mass spectrometry using electro spray ionisation and time of flight detection (Mariner System 572 Applied Biosystems, Foster City, CA). The conjugate solution was diluted to concentration of 10 μM with water and then further diluted by a factor of two with TRIS buffer (100 mM Tris-HCl, 20 mM NaN_3, 2 mM EDTA, 0.2% Tween-20, pH 8.0) and stored in a freezer (−20°C).

Fluorometry

The stock solutions of the oligonucleotide conjugates were diluted with a solution of TX-100 (0.1%, aq) to concentration of 125 nM. The fluorescence emission spectra of these solutions were recorded in wavelength range of 525–700 nm with in-house constructed spectrofluorometer which employed argon-krypton laser as illumination source. The excitation wavelength was 514 nm for BF523 and BF530 labels and 531 nm for TAMRA, BF545, BF560, and BF568 labels.

The labeled oligonucleotides (125 nM, 0.1% TX-100, aq) were pipetted into a 384-well microtitration plate (TC-grade with black walls and clear bottom, Greiner Bio-One, Frickenhausen, Germany) and measured with the ArcDia TPX Platereader (Arctic Diagnostics Oy, Turku, Finland) using liquid measurement mode and 10 s integration time. The measurements were carried out using six sample replicates. The results are summarised in Table II. The instrument was 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. The instrument was recently described in detail [27,36].

Coating of Microspheres

Microspheres (19.2 mg, 1×10^9 pcs) were washed twice with water (500 μl) and once with MES-buffer (100 mM, pH. 5.5) by sequential centrifuging (5000 g, 3 min) and resuspension. The final resuspension was done in MES buffer (50 μl). A fresh solution of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide EDAC (25 mM, 12.5 μl) in MES-buffer was added. Solution of capture oligonucleotide **3** in water (62.5 μl, 10, 20, 40, 100, 200 or 1000 μM) was added. The reaction mixtures were vortexed for 5 s in 1 min intervals during the first 10 min, and then left for incubation overnight under continuous shaking (1400 rpm; 22°C). The microsphere suspensions were transferred in to clean microtubes and the unreacted oligonucleotides were removed by sequential washing (7×500 μl) with TRIS buffer (50 mM Tris-HCl, 10 mM NaN_3, 1mM EDTA, 0.1% Tween-20, pH 8.0). After the final aspiration the microspheres were resuspended in 2 ml of the same buffer, and the microsphere concentration was determined with Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA).

Optimisation of Coating Density

Microspheres coated with oligonucleotide **3** were diluted to concentration of 1×10^4 pcs/μl with hybridization buffer (0.6 M NaCl, 50 mM Tris-HCl, 10 mM NaN_3, 1mM EDTA, 0.1% Tween-20, pH 8.0). A Solution of the TAMRA-labeled oligonucleotide **4**, complementary to the oligonucleotide **3**, was diluted with hybridization buffer to give solutions of 1.6, 6.6, 13.3, and 26.6 nM. 10 μl of the microsphere suspensions were then dispensed in the wells of 384-plate, in 3 replicates and the TAMRA-labeled oligonucleotide (10 μl, 1.6, 6.6, 13.3, and 26.6 nM) was dispensed to the wells. The plate was sealed with adhesive PCR film (Abgene, Surrey, UK), incubated overnight under continuous shaking (22°C, 1300 rpm) and measured with the ArcDia TPX Platereader using the particle measurement mode. In the 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 a 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 the results, the individual datapoints (microspheres) characterized with the focal duration time less that 5 msec were omitted.

Sandwich Hybridization Assay

The tracers (labeled oligonucleotides) were prediluted to concentration of 6 nM and the oligonucleotide **2** (analyte) was prediluted in the concentrations of: 0 pM, 39 pM, 98 pM, 246 pM, 614 pM, 1.54 nM, 3.84 nM, 9.6 nM and 24 nM (later called as analyte standards). All dilutions were made in hybridization buffer. The prediluted microsphere suspension (50 μM concentration of oligonucleotide in coating) and analyte standards were combined in 1 to 1-ratio to give microsphere-analyte cocktail. The cocktail (10 μl) was then dispensed in the wells of a 384-plate, in 6 replicates by Miniprep 60 autodispensor (Tecan; Maennedorf, Switzerland). Tracer (10 μl, 6 nM) was dispensed to the wells by the same dispensor automate. The plate was sealed with adhesive PCR film, incubated overnight under continuous stirring (22°C, 1300 rpm) and measured with the ArcDia TPX Platereader as described for oligonucleotide **4**.

RESULTS AND DISCUSSION

Labeling of Oligodeoxyribonucleotides

The labeling of amino modified oligonucleotides were performed by using typical labeling conditions [37]. The same conditions were used for all six labels. The

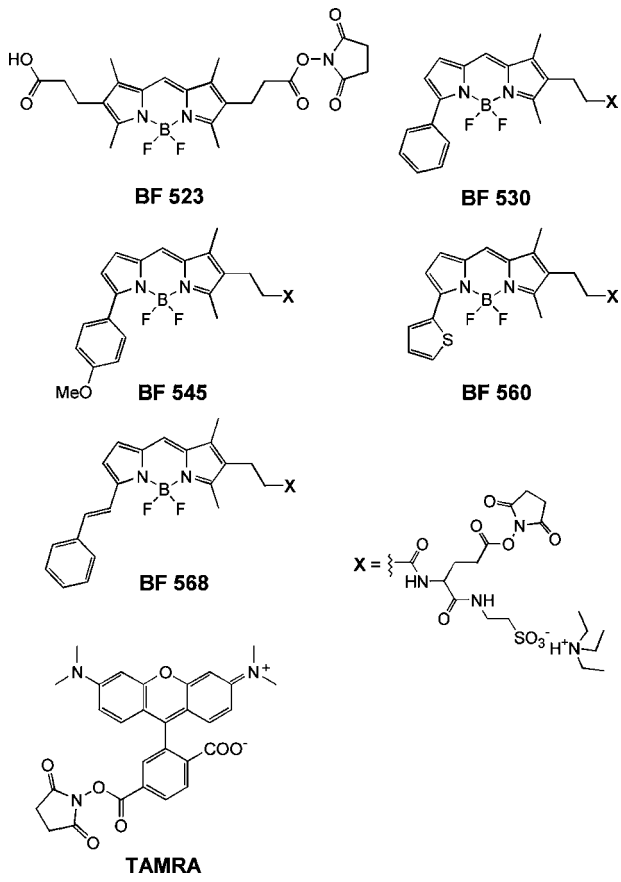

Fig. 1. Chemical structures of the labeling reagents.

chemical structures of the labeling reagents are shown in Fig. 1. The labeling efficiencies (ratio between labeled and unlabeled oligonucleotide) were surprisingly low (between 51 and 17%), although the labels were used in 20 fold molar excess, and both the label and the oligonucleotide were used in relatively high concentrations. The low labeling efficiency can be partly explained by electrostatic repulsion between the negatively charged label and the oligonucleotide. Oligonucleotides are also negatively charged compounds, and since the poorest labeling efficiencies were obtained with labels containing negatively charged sulfonyl group, the electrostatic repulsion may have effect on labeling reaction. Quite often low labeling efficiency, especially with hydrophobic labels, originates from low solubility of the labeling reagent to aqueous solutions and partial precipitation of the label upon labeling reaction. In this study the BF523 and TAMRA labels were the only labels where such partial precipitation was observed. These two labels, however, gave the highest labeling efficiencies. Thus in this case partial precipitation of the labeling reagent cannot be considered as the main reason for low labeling efficiency. Instead, the most probable explanation for the low labeling efficiency is that the ratio of labeled and unlabeled oligonucleotide was already biased by the step of ethanol precipitation before final chromatographic purification. The precipitation procedure probable favours precipitation of unlabeled oligonucleotide and thus resulted in biased labeling efficiency. However, it was found that without precipitation procedures non-covalent binding of the labels to oligonucleotides strongly interfered chromatographic purification and without ethanol precipitation, the HPLC purification was practically impossible to perform. The non-covalent label adducts dissociated during chromatography and resulted in multiple peaks and product fractions which all contained also the free label. On the other hand, after ethanol precipitation, the HPLC purification was easy to perform and the chromatogram showed practically only two peaks, the unlabeled oligonucleotide (retention time 2 min) and the label-oligonucleotide conjugate (retention times between 4.5 and 11 min). Thus the peaks of unlabeled oligonucleotide and label-oligonucleotide conjugates were readily distinguishable form each other. The HPLC data are presented in Table I. The retention

Table I. HPLC Data of the Labeled Oligonucleotides

Label	HPLC fraction collected (min)	Retention time (min)	Labeling efficiency (%)
BF523	6–7.5	6.50	43
BF530	9–10.5	9.49	17
BF545	9.5–11	9.79	38
BF560	8.5–10	9.12	41
BF568	10.5–12	10.96	36
TAMRA	4.5–6	4.56	51

times of the BF-labeled oligonucleotides correlate well with the size of the label. The retention time of the label-oligonucleotide conjugate with lowest molecular weight, labeled with BF523, was 6.5 min while the retention time of the conjugate with highest molecular weight, labeled with BF568, was 11 min.

Spectroscopy

The absorption and fluorescence data are summarized in Tables II and III. All the BF labels show decrease in absorptivity upon conjugation to oligonucleotides. The decrease was between 11 and 25% when compared to the unconjugated labels. Also, the absorption and emission maxima were red-shifted upon conjugation. The red-shift of absorption maximum was between 3 nm (BF523) and 5 nm (BF568), and of emission maximum between 3 nm (BF523) and 6 nm (BF560). Compared to IgG conjugates of the same labels [35] the oligonucleotide conjugates show more red-shifted absorption and emission spectrum. In case of IgG conjugates the red-shift of both absorption and emission maximum was only 1 to 2 nm.

Quantitative two-photon excited fluorometry was carried out with the Arcdia TPX platereader. Two-photon excited fluorescence data of the free labels [33,34] and the oligonucleotide conjugates are summarized in Table III. For the free labels highest TPE signal was obtained with the BF560 label and the lowest signal was obtained with the TAMRA label. In absolute units the highest TPE signal was obtained with the BF545 conjugate. Similar results were obtained in the study of BF-IgG conjugates where overall highest TPE signal was also obtained with the BF545 conjugate [35].

Upon conjugation to oligonucleotide all labels show decrease in fluorescence intensity. Decrease was smallest with the BF523 label (residual fluorescence 65%) and largest with the BF560 label (residual fluorescence 36%). The conjugation related quenching was thus strongest with the BF560 label. This label showed strong conjugation related quenching and self-quenching, also upon conjugation to IgG [35]. However, the BF560-IgG conjugate with low substitution degrees provided higher TPE signals than the BF545 conjugates and it was observed that as IgG conjugate the BF560 suffered stronger self-quenching than the other BF-labels. In case of oligonucleotide conjugate, where each oligonucleotide carries exactly one label molecule, self-quenching should not be present in theory, but the fluorescence decrease is a result of conjugation related quenching. Thus, comparison between BF-IgG and BF-oligonucleotide conjugates show that as an oligonucleotide conjugate BF560 is most prone to conjugation related quenching whereas as IgG conjugates the BF530 and BF545 are more prone than the BF560 to conjugation related quenching.

Microsphere Coating

It has been reported in the literature that too dense coating of oligonucleotides to the polymer surface prevents efficient hybridization of the complementary oligonucleotide to the coating oligonucleotide [38–40]. In order to ensure efficient hybridization and on the other hand to obtain microspheres with maximum binding capacity, the microspheres were coated with variable concentrations of aminomodified oligonucleotide **3**. The highest concentration of the oligonucleotide (500 μM) was calculated to be equal to the number of carboxylic acid residues at the surface of microspheres in coating suspension. In order to study the optimal coating density microspheres were titrated with commercial TAMRA labeled oligonucleotide **4** complementary to the coating oligonucleotide. The titration curves are presented in Fig. 2. The optimal coating density is mainly composed of two factors, binding capacity and hybridization efficiency. According to the results the highest concentration of coating oligonucleotide, i.e. microspheres with the highest coating density, provided lowest signal levels. This indicates that the density of oligonucleotides at the microsphere surface was too high for efficient hybridization. Too dense coating causes steric hindrance that prevents efficient hybridization of the complementary oligonucleotide to the coating oligonucleotide. Another factor that may have an effect on hybridization efficiency is electrostatic repulsion between the anionic phosphate moieties. Lowering of the coating density resulted in increase in hybridization efficiency and the highest hybridization efficiency was obtained with microspheres coated with oligonucleotide of 50 μM concentration, a concentration that corresponds 1/10 of the amount of carboxylic acid residues on the surface of microspheres. Coating with concentrations lower than 50 μM resulted again lower signal levels. At this time

Table II. Absorption Data of Free Labels and Conjugates Measured from 0.1% TX-100 (aq) solutions

Label	λ_{abs} Free label [nm][a]	λ_{abs} Conjugate [nm]	ε Free label [cm^{-1} M^{-1}][a]	ε Conjugated label [cm^{-1} M^{-1}]	CF (A_{260}/A_{max}) [a.u.]
BF523	524	527	75 000	56000	0
BF530	533	536	54 000	48000	0.05
BF545	547	550	64 000	56000	0.10
BF560	565	569	69 000	59000	0.05
BF568	574	579	118 000	105000	0.05
TAMRA	547 (MeOH)[b]	561	91 000 (MeOH)[b]	102000	0.30[b]

[a] Data from ref. [33,34].
[b] Data from ref. [37].

the low signals can be accounted to result from low binding capacity rather than from low hybridization efficiency. These results are in good agreement with the previously reported results [38–40].

Hybridization Sandwich Assay

Performance of the label-oligonucleotide conjugates was tested in a separation-free hybridization 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 nucleic acid hybridization assay (Fig. 3.), bioaffinity complexes, consisting of a capture oligonucleotide (2), the analyte oligonucleotide (3) and a fluorescent tracer oligonucleotide (4), are formed on the surface of individual microspheres (1). 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 of bioaffinity complexes on the surface of a microsphere. The optical configuration of the fluorometer and the physical phenomena related to the measurement process have been described in detail in our previous publications [27,36].

The model sandwich hybridization assay was performed using synthetic oligonucleotides. The sequences of oligonucleotides utilized were based on the previously published prostate spesific antigen (PSA) – coding mRNA sequence [41]. The assays were incubated overnight in order to ensure equilibrium binding. The assays were done in six replicates to study the signal variation between the different label conjugates. According to the results all the conjugates show excellent assay response (Fig. 4.). Saturation of the microsphere surface and simultaneous extinction of the tracer can be seen as hook-effect in the assay curves. All the conjugates show a hook at the same analyte concentration. The signal levels of the negative standards are in accordance with the results obtained in the solution measurements, i.e. fluorescence signal obtained from the microspheres are of same level as liquid signals. This indicates that none of the label conjugates show significantly stronger unspecific binding than the others. Also, since the signal levels of the negative standards are low overall it can be concluded that the fluorescence originates from the free tracer in solution rather than from unspecific binding.

The assay curve of the best BF-oligonucleotide conjugate, BF523 conjugate, is presented in Fig. 5. The BF523 conjugate gives highest fluorescence signal, best signal-to-noise ratio and also highest assay sensitivity when

Table III. Fluorescence Data Measured from 0.1% TX-100 (aq) Solutions

Label	λ_{max} Fl label [nm][a]	λ_{max} Fl conjugate [nm]	TPE label [a.u.][c]	TPE conjugate [a.u.]	Residual fluorescence (%)
BF523	533	536	65	42	65
BF530	557	561	183	75	41
BF545	574	579	207	106	51
BF560	579	585	250	91	36
BF568	586	588	188	87	46
TAMRA	573[b](MeOH)	583	50	25	50

[a] Data from ref. [33,34].
[b] Data from ref [37].
[c] Data from ref. [35].

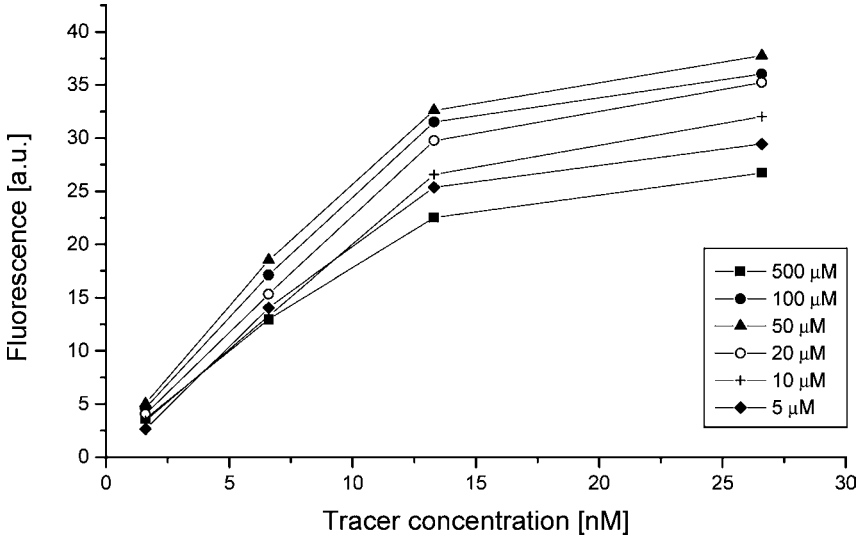

Fig. 2. Hybridization efficiency with different coating densities.

calculated by the 3SD principle. However, the difference in signal levels and also in assay sensitivity between the three best BF conjugates, BF523, BF530 and BF560 conjugates, is small (Table IV). Compared to the study of BF labels in immunometric assay [35], significant differences can also be found in the performance between the BF labels. As oligonucleotide conjugate the assay performance of the BF523 label is clearly better than would be expected on the basis of the results obtained with the IgG conjugates. Also, when looking at the fluorescence signals of the oligonucleotide conjugates obtained from solution measurements, the assay performance of the BF523 conjugate is unexpectedly good. Another interesting observation relates to the assay performance of the BF545 conjugate. The fluorescence signal obtained from the BF545 conjugate in assay conditions is not as high than it would be expected based on solution measurements. The same phenomenon was observed also with the IgG conjugates of this label. Overall, in hybridization assays the differences between BF labels are smaller than in immunometric assays. This is understandable since in case of oligonucleotide conjugate each oligonucleotide carries exactly one label molecule and the effects relating to unlabeled product, multiple labels, self-quenching and inactivation of the tracer do not interfere the tracer performance.

Comparing the results to those obtained from the study of the BF labels in immunometric assay [35], the most prominent difference is the performance of TAMRA as a label. In this study the difference in signal levels between the best BF label and TAMRA was 2 fold whereas in immunometric assay the difference in signal levels was 8 fold. This suggests that TAMRA is better suited for labeling of oligonucleotides than proteins and also that in labeling of oligonucleotides the hydrophobicity of the label is not so deleterious. However, when looking the average CV's and the lowest limit of detection, the BF labels provide better sensitivity and lower assay variation than TAMRA as a reporter (Table IV). The CV profile was flat with all labels and the average CV was between 5.1% (BF545) and 8.7% (TAMRA).

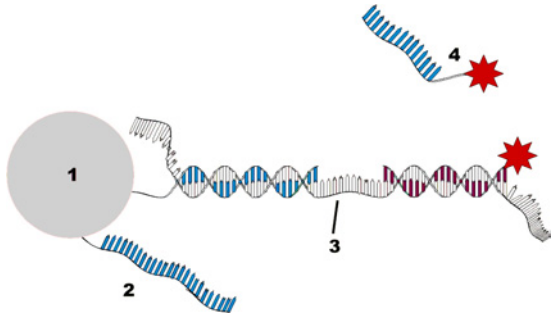

Fig. 3. Principle of nucleic acid hybridization assay.

CONCLUSIONS

In this paper, the use of dipyrrylmethene-BF_2 fluorophores as labels for nucleic acid hybridization assay has been studied. The label conjugates show minor changes in absorption and emission spectra when compared to those of unconjugated labels. The fluorescence

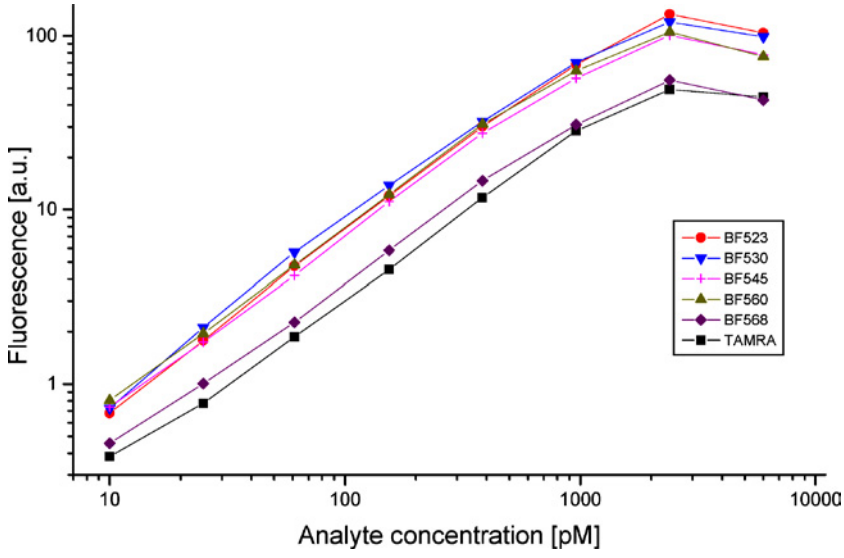

Fig. 4. Standard curve overlay.

quantum efficiency of the labels, however, is markedly decreased upon conjugation to oligonucleotide. The conjugation related quenching was found to be strongest with thienyl substituted dipyrrylmethene-BF_2 label (BF560), and weakest with alkyl substituted BF523 label. Conjugation related quenching did not correlate with hydrophobicity or bulkiness of the label, as it was the case with the IgG conjugates of the same labels. This finding suggests that the quenching mechanisms are different in case of oligonucleotide and IgG conjugates.

The oligonucleotide conjugates were tested as tracers for a separation free hybridization assay using the ArcDia TPX assay technique. The results show that, all labels provide standard curves with dynamic range of 2–3 orders of magnitude and limit of detection between 4 and 12 pM. In assay conditions, all the dipyrrylmethene-BF_2

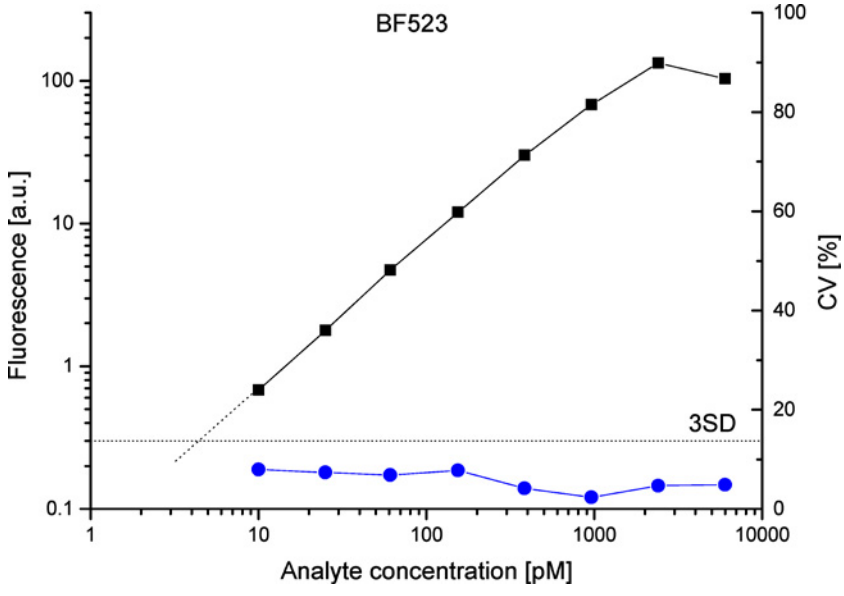

Fig. 5. Standard assay curve using BF523 labeled oligonucleotide, 3SD level of negative control and CV.

Table IV. Hybridization Assay Data

Label	Signal of the negative standard (A.U.)	3 SD of the negative standard	Average CV (%)	Calculated sensitivity (pM)	S/B ratio (c = 960 pM)
BF523	1.61	0.3	6.20	4	43
BF530	1.89	0.33	5.82	4	38
BF545	2.59	0.4	5.10	6	23
BF560	2.06	0.32	5.18	5	32
BF568	1.80	0.29	5.46	12	18
TAMRA	1.08	0.28	8.71	9	27

fluorophores gave higher signal levels than the conventional TAMRA label. Of the five dipyrrylmethene-BF_2 labels included in this study, alkyl substituted BF523 and phenyl substituted BF530 labels provided the best performance in nucleic acid hybridization assay. With these labels the lowest limit of detection was 4 pM. This detection limit is considered excellent for a separation free assay method, especially when taking into account that optimisation of assay parameters was not carried out. Furthermore, the results show that the performance of the label in hybridization assay 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 the microsphere surface and for the labels in liquid phase.

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