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Set of fluorochromophores in the wavelength range from 450 to 700 nm and suitable for labeling proteins and amino-modified DNA

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Abstract

We describe the synthesis, purification, and spectral properties of new dyes and reactive labels. They absorb in the visible range between 450 and 700 nm and display analytically useful fluorescence. They were made amino-reactive by esterification with *N*-hydroxysuccinimide (NHS). The resulting oxysuccinimide (OSI) esters were covalently linked to the amino groups of human serum albumin (HSA) or certain DNA oligomers. Except for dyes **9** and **13**, they contain one reactive group only in order to avoid cross linking of biomolecules. Labeling of amino-modified biomolecules was performed by standard protocols, and the labeled proteins and oligonucleotides were separated from the unreacted dye by gel chromatography using Sephadex G25 as the stationary phase in the case of proteins, and reversed-phase HPLC in the case of DNA oligomers. The dyes also have been used as donor–acceptor pairs in fluorescence energy transfer systems and in energy transfer cascades.
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1. Introduction

Fluorescence spectroscopy and time-resolved fluorescence have become firmly established and are now widely applied in biophysical studies, in environmental monitoring, clinical chemistry, DNA sequencing and gene analysis. Fluorescent dyes labelled to proteins or DNA oligonucleotides have attracted particular interest. Fluorescence detection is routinely used in immunoassays, hybridization assays, enzyme-based assays, sequencing and other analytical procedures. Fluorescent markers are used for cell identification and flow cytometry, and in cellular imaging to reveal the localization and movement of intracellular substances by means of fluorescence microscopy. However, in the final analysis, wavelength and time resolution required of the instruments are determined by the spectral properties of the fluorophores [1,2].

Biological material has an intrinsic fluorescence (e.g. from aromatic acids, flavins or NADH) in the short wavelength range and with short decay times [3]. However, labeled biomolecules having fluorescence outside the biological and background fluorescence are preferred in analytical practice. The design of new labels is determined by the spectral properties of the material to be probed. There are two ways to circumvent this problem. One possibility is to use

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long-lived (>100 ns) dyes. In this case, the detection gate is opened after a certain decay time after which the intrinsic fluorescence of biological material has vanished. The other possibility is to label the material with chromophores having much longer excitation and emission wavelengths than the background.

The autofluorescence of biological samples is particularly strong at <500 nm, and particularly weak at >600 nm, except for the porphyrins. Numerous fluorescent labels for proteins or DNA are available in the 300–500 nm range, but all of them suffer from either poor photostability, pH-dependence, low quantum yields, toxicity, or inappropriate decay time.

Few fluorescent probes exist which absorb in the red or near-infrared region and even fewer are available in a reactive form [4–6]. Cy5 is a popular reactive cyanine label absorbing and emitting in the NIR. Several other cyanine dyes and squaraines are known [7–9] and can be obtained as both asymmetrical and symmetrical labels. Large variations in the spectral properties are possible by variation of the heterocyclic moieties. Long-wavelength probes can be excited by inexpensive light sources such as diode lasers which represent a convenient source of monochromatic intense light and are quite affordable [8].

We present a set of new labels having the features of: (a) covering a wide spectral range, (b) a wide range of decay times, and (c) in many cases having low quantum yield in the unconjugated form, but high quantum yields if linked to a biomolecule. They have been characterized by spectra and quantum yields both in the free and conjugated form.

2. Experimental

2.1. Material and methods

2.1.1. Chemicals, proteins and buffers

Human serum albumin (HSA), anti-HSA and Sephadex G25 were purchased from Sigma (Steinheim, Germany). Silica gel 60 (40–63 μm) RP-18, silica gel 60 (63–200 μm) and all other chemicals and solvents were from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). The quaternized indoles in the synthesis of the squaraines and cyanines were synthesized following a procedure described recently [5,9].

Phosphate buffer, pH 7.2, 100 m*M*, was obtained by dissolving 3.17 g of $NaH_2PO_4 \cdot H_2O$ and 13.7 g of $Na_2HPO_4 \cdot 2\ H_2O$ in 1 liter of doubly distilled water. Bicarbonate buffer, pH 9.0, 50 m*M*, was prepared by dissolving 2.1 g of $NaHCO_3$ in 500 ml of doubly distilled water. The desired pH was adjusted with 1 N HCl or 1 N NaOH, respectively. All chemicals were of analytical grade and used as received.

2.1.2. Chromatography

Analytical thin-layer chromatography (TLC) was carried out on plates from Merck. Reversed-phase (RP-18 F_{254}) aluminium sheets (thickness 0.2 mm) were used for polar solvents, and silica gel 60 (thickness 0.2 mm) for nonpolar solvents. Gel permeation chromatography was carried out using Sephadex G25 (medium) as the stationary phase in a 20-cm glass column of 1 cm I.D., and a 22 m*M* phosphate buffer, pH 7.2, as the eluent.

2.2. Syntheses

Fig. 1 gives the chemical structure of the dyes synthesized. In this context we refer to *dyes* as the unreactive forms which usually are the precursors of the respective *labels* (the amino-reactive form).

In the following, a short sketch is given of the synthetic routes that lead to the new dyes (chromophores). All dyes were purified via column chromatography (CC) (20×2 cm) using reversed phase silica gel as the stationary phase and a methanol/water mixture as the eluent, except for dye **7** which was purified by reversed-phase medium pressure liquid chromatography (MPLC). Dye **10** was purified using MPLC with silica gel 60 as the stationary phase and an acidic methanol/chloroform mixture as the eluent, while dye **13** was purified by CC using silica gel as the stationary phase and a chloroform/methanol mixture as the eluent. All structures were confirmed by mass spectrometry and NMR spectroscopy.

2.2.1. Dye 1

The ligand 5-carboxy-2,2′-bipyridine was attached to ruthenium-bisbipyridyl dichloride by reacting the

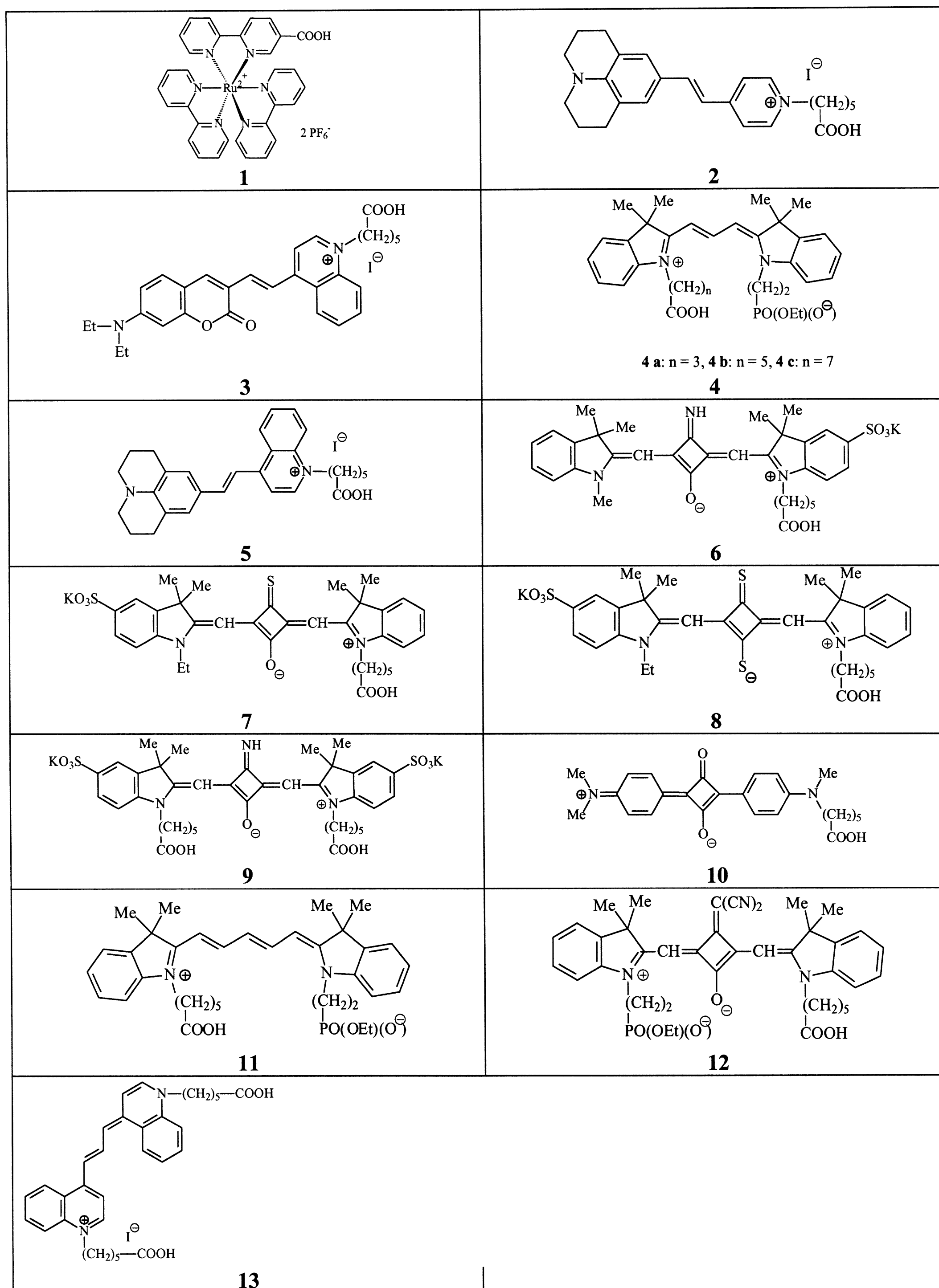

Fig. 1. Overview of the chemical structures of the synthesized dyes.

two components (in 1:1 molar ratio) in a methanol/water mixture at 80 °C for 24 h by analogy to a method described [10,11]. The dye precipitated from an acidic aqueous solution by adding NH_4PF_6. The yield was about 73% of fine purple crystals of melting point >330 °C.

2.2.2. Dye 2

The starting material (9-formyljulolidine) was reacted with one molar equivalent of 1-(5-carboxypentyl)-γ-picoline bromide in methanol and piperidine following a procedure described earlier [5,12]. Then, the solvent was removed and the dye purified by CC. The yield was about 20%.

2.2.3. Dye 3

Condensation of 7-diethylamino-2-oxo-2H-chromene-3-carbaldehyde with 1-(5-carboxypentyl)-lepidine bromide in methanol and piperidine was carried out by analogy to a method described in the literature [13,14]. The raw dye was purified via CC. The yield was about 65% of violet crystals.

2.2.4. Dye 4a

This asymmetrical cyanine dye was synthesized by reacting equimolar quantities of 1-(3-carboxypropyl)-2,3,3-trimethyl-3H-5-indolium bromide and 1-[2-(diethoxyphosphoryl)ethyl]-2, 3, 3-trimethyl-3H-indolium bromide with 1,1,1-trimethoxymethane by analogy to a synthesis described recently [15]. The yield was about 10% of a red powder. The dyes **4b** and **4c** were synthesized in complete analogy using starting materials with different lengths in the side chain of the indolium bromide. The reactive oxysuccinimide ester of **4c** is more stable than the OSI esters of **4a** and **4b**.

2.2.5. Dye 5

Commercial 9-formyl-julolidine was reacted with one molar equivalent of 1-(5-carboxypentyl)-4-methyl-quinoline bromide in methanol and piperidine analogous to a literature procedure [5,12]. After the solvent was removed, the dye was purified by CC. The yield was about 25% of pure crystals.

2.2.6. Dye 6

This asymmetrical dye was synthesized in a one-step reaction. 1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-5-indoliumsulfate, 1,2,3,3-tetramethyl-3H-indolium iodide, and the amino squaric acid were dissolved in equimolar parts in acetic anhydride and refluxed for 10 h as described for other dyes [15]. After removing the solvent, the dye was purified by CC. The yield was 10% of blue crystals.

2.2.7. Dye 7

One oxygen in the squarylium moiety [15] was replaced by dissolving the dye in pyridine adding a large excess of phosphorus pentasulfide. The reaction mixture was refluxed for 6 h. After removing the solvent, the residue was dissolved in a little water and refluxed for 30 min. It was precipitated from diethylether and was purified by CC. The yield was about 22% of a green powder.

2.2.8. Dye 8

This dithiosquarylium dye was prepared by double thionation of an asymmetrical squarylium dye. Specifically, the non-thiolated dye [15,16] and two molar amounts of Lawesson's reagent (from Aldrich) were suspended in a mixture of *p*-xylene and hexamethylphosphoramide (from Aldrich). The suspension was refluxed for 5 h [17]. The mixture was evaporated and purified. The yield was about 38% of a green powder.

2.2.9. Dye 9

This symmetrical squarylium dye was synthesized by dissolving two equivalents of 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-5-indoliumsulfate and one equivalent of amino squaric acid in acetic anhydride and refluxing the reaction mixture for 14 h as described for other dyes [15]. The solvent was removed and the dye purified by CC. The yield was about 10% of pure blue crystals.

2.2.10. Dye 10

N-Methyl-*N*-(carboxypentyl)aniline, *N*,*N*-dimethylaniline and squaric acid were dissolved in propanol, and 1,1,1-trimethoxymethane was added and the reaction carried out under a nitrogen atmosphere at 70 °C similar to the method described in Refs. [18,19]. Volatiles were removed and the dye was separated from by-products using MPLC. The

yield was about 19% of a blue-green powder with a melting point of >230 °C.

2.2.11. Dye 11

This asymmetrical cyanine dye was synthesized by condensing equimolar amounts of 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-5-indoliumbromide, 1-[2-(diethoxyphosphoryl)ethyl]-2,3,3-trimethyl-3H-indolium bromide and 1,1,3,3-tetramethoxypropane in pyridine for 14 h. We used tetramethoxypropane rather than the 1,1,3-trimethoxypropene used in Ref. [20]. The solution was then triturated with diethylether on which the dye precipitated. The yield was about 10% after purification by CC.

2.2.12. Dye 12

Equimolar quantities of 1-[2-(diethoxyphosphoryl)ethyl]-2,3,3-trimethyl-3H-indolium bromide, 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-5-indoliumbromide and dicyanomethylensquaric acid [21,22] were dissolved in a butanol/toluene mixture and refluxed for 18 h as described for other cases [23–25]. The solvent was removed and the dye was separated from the by-products by CC. The yield was about 10% of golden-colored crystals.

2.2.13. Dye 13

This symmetrical cyanine dye was synthesized by refluxing two equivalents of 1-(5-carboxypentyl)-lepidine and one equivalent of 1,1,1-trimethoxymethane in pyridine for 2 h. The reaction mixture was triturated with diethylether whereupon the dye precipitated [26,27]. The bis-reactive dye was purified by CC. The yield was about 18% of blue crystals.

2.3. Activation of dyes with N-hydroxysuccinimide to obtain reactive labels; general procedure

The respective dye was dissolved in either acetonitrile, dimethylsulfoxide or dimethylformamide. Equimolar amounts of dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were added and the reaction mixtures slowly stirred in the dark and at room temperature. The solution was filtered and the solvent removed. The OSI esters were characterized by thin-layer chromatography.

2.4. Labeling of proteins or amino-modified DNA ("bioconjugation")

Proteins and peptides usually contain a number of reactive side chains such as ε-amino groups of lysine and the α-amino groups of the *N*-terminal amino acids. Reactive esters, especially oxysuccinimide (OSI) esters are among the most used reagents for labeling amino groups. Amino-modified oligonucleotides may also be labeled using OSI.

2.4.1. Labeling of proteins; general procedure

One milligram of protein or peptide was dissolved in 0.5 ml bicarbonate buffer of pH 9 and typically 0.1–0.3 mg of the label is added. Labeling takes place within 1 h in the dark and at room temperature. The labeled protein can be separated from unreacted dye by size-exclusion chromatography using Sephadex as the stationary phase and phosphate buffer of pH 7 as the eluent.

2.4.2. Labeling of amino-modified oligonucleotides; general procedure

Amino-modified 15-mer oligonucleotide (0.30 μmol) was dissolved in 500 μl of bicarbonate buffer. Then 2 mg of label (OSI ester) was added and the solution allowed to stand for 15 h in the dark; 1.5 ml of ice-cold ethanol was added. The solution was mixed well and placed at −18 °C for 1–2 h. The solution was then centrifuged at 15 000 rpm for 10 min. The supernatant containing unreacted dye was removed carefully and the pellet containing labeled and unlabeled oligonucleotide rinsed twice with ice cold ethanol.

Labeled oligonucleotides were purified by HPLC using a Hibar pre-packed column RT (250×4 mm) packed with LiChrosorb RP 18 (10 μm). The rinsed pellet was dissolved in 0.1 *M* triethylammonium acetate (TEAA), pH 7.1. The solution was loaded onto the column in 0.1 *M* TEAA and a linear 10–65% acetonitrile gradient was run over for 30 min. The unlabeled oligonucleotide migrated fastest, followed by the labeled oligonucleotide. Finally, the free dye-acid along with the OSI ester were eluted [28]. The solvent of the HPLC fraction was removed on a rotary evaporator. The residue was dissolved in 100 μl of water and 1 ml of ice-cold ethanol was

added. The turbid solution was mixed well and placed at −18 °C for 1–2 h. The solution was centrifuged at 15 000 rpm for 10 min. The supernatant was removed carefully and the pellet containing the labeled oligonucleotide either stored at 4 °C or dissolved in 22 m*M* PBS for further experiments.

3. Results and discussion

Table 1 lists the dyes presented here according to their absorption maxima and gives absorption and emission spectra as determined in phosphate-buffered saline (PBS), pH 7.2. Generally, the first step in the design of an effective label is to identify and synthesize a chromophoric system that matches the desired wavelength. The next step is to introduce a reactive group capable of coupling to an amino group under moderate conditions.

It is important that the labels are satisfactorily soluble in aqueous solution. In certain cases, sulfo- and phosphonic acid groups were introduced (dyes **4**, **6**, **7**, **8**, **9**, **11**, **12**) to improve the solubility in water and to reduce aggregation of the label. Secondly, a small molecular size of the label (compared to the biomolecule) is desirable in order not to compromise biological activity. Labeling is usually carried out in aqueous buffer at pH 9 at room temperature within 1 h.

A reactive label has to have a reactive group that enables covalent conjugation to the target. Covalent attachments are preferred due to the higher stability during the analysis and their higher specificity [29,30].

The most common groups to be labeled in aqueous medium are the amino group and the thiol group. Coupling to functional groups such as carboxy, hydroxy, keto or aldehyde groups is less common. For labeling amino groups, the oxysuccinimide method is most common and schematically outlined in Fig. 2 [31].

3.1. The ruthenium label 1

The absorption of this label matches both the 488-nm emission of the argon ion laser and the blue LED. Its Stokes shift is extraordinarily large (222 nm), even larger than that of other ruthenium labels [10] which enables an easy separation of excitation and emission light. Generally, the lifetimes of the ruthenium labels are very long (between 200 ns and 2 μs). The label is monoreactive and this prevents crosslinking.

Table 1
Absorption and emission maxima of the dyes presented in this work

Dye no.	Acronym	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	ε at λ_{max} (l mol^{-1} cm^{-1})	QY	QY of conjugates
1	5MC	453	675	10 000	<0.01	Up to 0.01
2	FR495	495	636	20 000	<0.01	Up to 0.41
3	FR521	520	700	35 000	0.006	Up to 0.6
4c	FO545	545	562	86 000	0.04	Up to 0.3
5	FR568	568	670	30 000	<0.01	Up to 0.4
6	FR626	626	636	150 000	0.02	Up to 0.2
7	FR628	628	641	n.d.	n.d.	n.d
8	FR631	631	641	n.d.	n.d.	n.d.
9	FR632	632	645	210 000	0.057	Up to 0.34
10	PB642.6	642	666	75 000	0.04	Up to 0.52
11	FR642	642	663	170 000	0.17	Up to 0.25
12	FR662	662	682	130 000	0.02	Up to 0.2
13	PB710	701	718	210 000 (in methanol)	0.007	Up to 0.3

The dyes are listed according to their wavelength of absorption. Also given are the molar absorbances and quantum yields (QYs) of the free forms and the HSA-conjugated forms (both in PBS solution); n.d., not determined.

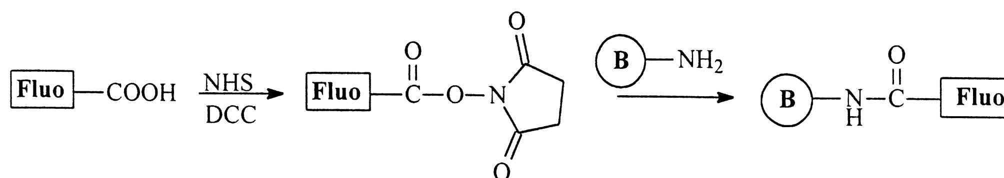

Fig. 2. The oxysuccinimide method for labeling amino groups. *Fluo*, fluorophore; *B*, biomolecule.

3.2. The cyanines and squaraines

The cyanines and squaraines were chosen as a group of long-wavelength absorbing chromophores because of their high photostability and their relatively small size. Their color can be easily adjusted to the desired wavelength (between 550 nm and 710 nm) by varying the heterocyclic nucleus or the number of double bonds in the polymethine chain. Compared to the cyanines, the squarilium dyes are even more fluorescent, probably because of their planarity and rigidity.

Compared to the absorption maxima, the emission maxima are shifted only by about 20 nm. Activation of the carboxy group to an OSI ester enables coupling to amino groups of biomolecules. Even though the effort to synthesize monoreactive dyes is substantial in these cases, the presence of only one reactive group in a dye avoids crosslinking. The labels exhibit molar absorbances of up to 220 000 l mol^{-1} cm^{-1}. The symmetrical dyes even have a higher molar absorbances (compare **6** with **9**). The quantum yield of the free dye is very low (less than 0.1) and increases when bound to the target [32].

3.3. Coumarin labels

The coumarins represent another extensively investigated group of fluorescent labels. They display large Stokes shifts (60 to 100 nm), a high photostability and good chemical stability. The quantum yield and the molar absorbance of **3** are moderate, though.

3.4. Styrylium labels

These labels (**2** and **5**) have a relatively low molar absorbance and a very low quantum yield in aqueous solution, but show a very high quantum yield if bound to the target.

3.5. Assessment of labels

The labels reported so far are different in terms of wavelength and decay times. Label **1** is particularly useful for time-resolved (gated) measurements in the μs time domain. It is also useful for polarization immunoassay of large proteins. It suffers, however, from some quenching by molecular oxygen and a moderate quantum yield (QY).

All other dyes have much shorter decay times, typically between 0.3 and 0.7 ns. Their spectra cover the whole visible range. At first glance, their QY appear to be low (Table 1). However it can be seen that QYs are mostly above 0.3 if conjugated to HSA. This, in fact, is advantageous since the detection system mainly sees the conjugate, but not the free label.

3.6. Stability of dyes and labels

All dyes are rather stable in solid state if stored at 4 °C. The corresponding labels are sensitive to humidity even in the solid state. Solutions of the labels in dimethylformamide lose activity over time and should not be used if older than 1 day. Solutions of the labels in buffer of pH 7 even decompose within a few hours.

3.7. Effect of conjugation on spectra

It is often assumed that the spectrophysical properties of a dye or a label remain unchanged on conjugation, but this is usually not the case. Spectral maxima (in absorption and fluorescence), decay times, and, in particular, quantum yields may be

substantially affected. For example, the absorption maximum of **11** shifts from 642 to 648 nm, and the emission to 666 nm upon covalent linkage to HSA. The absorption maximum of **4c** on conjugation shifts from 545 to 553 nm, and the emission to 564 nm.

It is known for most dyes that they can bind, in a non-specific manner, to proteins [24,25,33]. This is accompanied by an increase in fluorescence intensity. This is also true for most of the dyes presented here. On the other hand, it should be kept in mind that non-specifically bound dye/protein-aggregations will be separated during both gel chromatography and HPLC.

3.8. Effect of the dye-to-protein ratio (DPR)

The emission maxima of the **11**/HSA conjugates of varying DPR (0.6–5.4) were examined to prove the effect of covalently coupled HSA on the fluorescence intensity. Fluorescence intensities of solutions with a constant concentration of HSA (1.7 μ*M*) were measured. Fig. 3 shows the plot of the fluorescence intensities at the emission maximum versus the DPR. It can be seen that at a DPR of about 1, the optimum is reached. If more than one label is bound to the protein, the dye undergoes self-quenching. Even at high DPR, we have not observed any protein precipitation, despite the fact that some of the labels are only moderately hydrophilic.

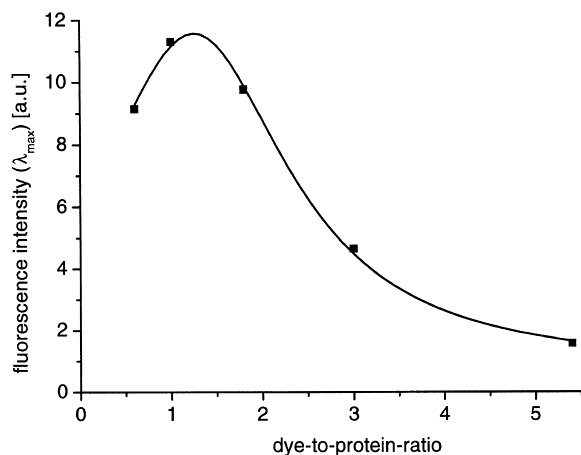

Fig. 3. Plot of the fluorescence intensities of **11**/HSA conjugates at the emission maximum versus the dye-to-protein ratio. [HSA]= 1.7 μ*M*.

3.9. Quantum yields and effects of conjugation

The quantum yield of the free **4c** in phosphate-buffered saline of pH 7 (PBS) is 0.04 and rises to 0.08 when the dye is conjugated to HSA at a dye-to-protein ratio (DPR) of 1.7. The quantum yield is 0.09, if **4c** is covalently bound to anti-HSA (DPR= 6.0). The highest quantum yield measured (0.31) is reached by attaching **4c** covalently to an amino-modified 15-mer oligonucleotide. In this case, the absorption maximum shifts to 550 nm, and the emission maximum to 565 nm. The emission spectra of **4c** in its free form and some of its conjugates are shown in Fig. 4.

3.10. Immunobinding study using fluorescence resonance energy transfer (FRET)

Label **4c** (the ‘‘donor’’) was used to mark human serum albumin (HSA), and **11** (the ‘‘acceptor’’) was used to label anti-HSA. The fluorescence intensity of the donor decreases to 40% upon addition of a fourfold excess of **11**/anti-HSA (Fig. 5; 1:4) compared to the intensity without acceptor (Fig. 5; 1:0). The fluorescence intensity of the acceptor (Fig. 5; 1:4) increases more than eightfold compared to the fluorescence of **11**/anti-HSA without donor (Fig. 5; 0:4). It is obvious that FRET from **4c** to **11** is very efficient.

FRET immunoassays pave the way for two-wave-

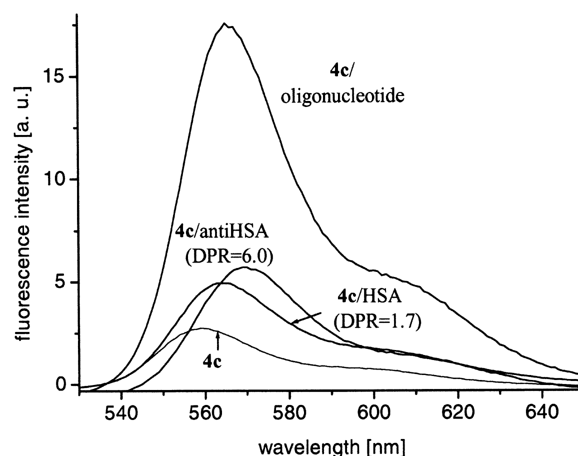

Fig. 4. Fluorescence spectra of **4c**, **4c**/HSA (DPR=1.7), **4c**/anti-HSA (DPR=6.0), and **4c**/oligonucleotide. λ_{exc}=500 nm.

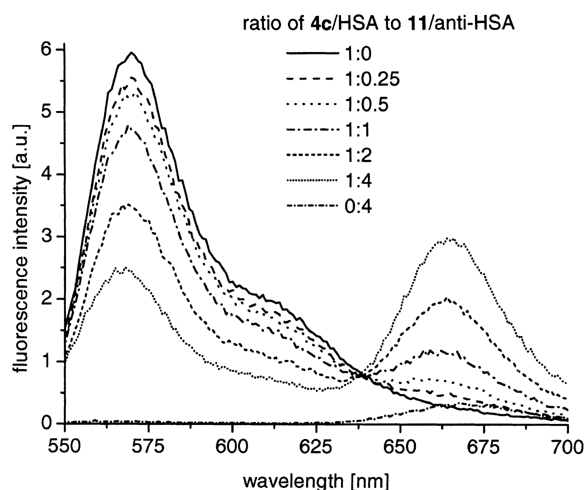

Fig. 5. Energy transfer study in which **4c**/HSA (DPR=1.7) of constant concentration was titrated with **11**/anti-HSA (DPR=5.9) (λ_{exc}=520 nm). [HSA]=0.1 μ*M*.

length immunoassays which are intrinsically self-referenced since the signal is calculated from the ratio of the intensities measured at two wavelengths. A plot of the ratios of the fluorescence intensities at 664 and 568 nm versus the ratios of the concentration of **11**/anti-HSA and of **4c**/HSA results in a sigmoidal curve (Fig. 6). At a fourfold excess of **11**/anti-HSA, saturation is reached. HSA concentrations can be determined in this way even in nanomolar concentrations.

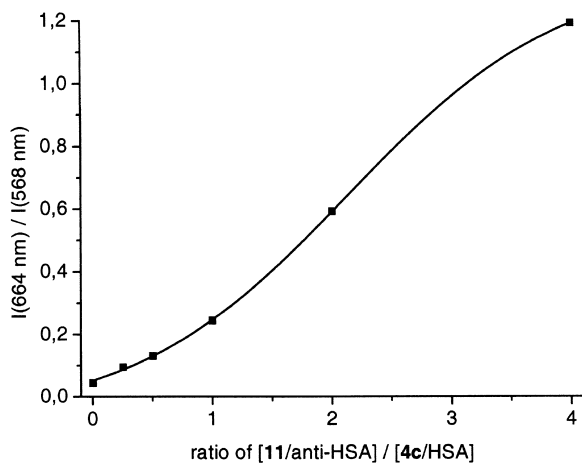

Fig. 6. Plot of the ratios of the fluorescence intensities at 664 and 568 nm versus the ratios of the concentrations of **11**/anti-HSA and the concentration of **4c**/HSA.

4. Conclusions

We introduce a set of fluorescent dyes matching the wavelength range from 450 to 700 nm. All dyes were activated to give amino-reactive OSI esters that can be covalently linked to proteins and amino-modified oligonucleotides. Most of the labels have a low quantum yield in the unconjugated form, but a high quantum yield when bound to a target. All dyes are promising labels for use in fluorescence resonance energy transfer (FRET) studies.

Acknowledgements

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