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ORIGINAL CONTRIBUTION

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Polymeric fluorescent dyes for labeling of proteins and nucleic acids

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Abstract In order to increase the sensitivity of fluorescence labeling in biochemical reactions and diagnostic procedures a labeling technique with polymeric fluorescence dyes was established and tested for its applicability. The fluorescence dye is based on the fluorophor coumarine and was covalently linked to the model proteins strepavidine and IgG. The dye was synthesized by radical polymerization of three different types of functional monomers to ensure water solubility, covalent coupling to proteins, and fluorescence. The molecular weight range was between 20 and 200 kDa. Fractions of narrow molecular weight distribution were prepared by gel filtration on Superdex 200. The relationship between size and charge of the different fractions was analyzed by gel electrophoresis. Covalent conjugation to proteins was carried out by formation of a peptide bond between a carboxylic group of the functional monomers and an amino group of the protein mediated by 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC). A novel type of gel electrophoresis was developed in order to analyze and optimize the conjugation reaction; the results were in agreement with those from analytical ultracentrifugation with fluorescence detection. Hydrodynamic studies of the uncoupled dye and the protein-dye conjugates exhibited a drastic decrease of Stokes radius of the dye due to the coupling to the protein. Under optimum conditions the fluorescence intensity of a protein-polymeric dye conjugate was enhanced 40-fold compared to a monomeric dye. Biotin binding to the protein streptavidin was not affected significantly by the conjugation with the polymeric dye. At present, the applicability of the polymeric dye in biochemical and diagnostic reactions seems to be limited due to strong but unspecific hydrophobic interactions which might be overcome by using fluoresceine as monomeric dye.

Key words Fluorescence labeling – polymer synthesis – coumarine – fluorescence detection – analytical ultracentrifugation

Introduction

Fluorescence dyes attached to biological molecules are widely used as labels in biochemical reactions, as reactants in diagnostic procedures and as stains in imaging biological structures. Those dyes fullfill high demands with respect to sensitivity and selectivity. Well-known examples are immunoassays [1], flow cytometry [2], *in situ* hybrid-

izations to chromosomes [3], automatic DNA-sequencing [4] and many more. In most cases proteins were labeled at primary amino groups with rhodamine- or fluoresceinisothiocyanate (FITC) [5, 6], and for nucleic acids modified nucleotides were used for the synthesis [7] or non-covalent labeling was achieved with ethidiumbromide [8]. The fluorescence dyes are monomers with only one fluorophor per molecule. In a few cases several fluorophor molecules could be attached to different sites of the same biological compound [9]. All those labeling techniques are limited in their sensitivity by the properties of the dye, because only one or a very limited number of dye molecules could be attached to the compound of interest. A new and conceptually simple strategy would be to synthesize polymeric fluorescence dyes with a high number of fluorophors per polymer and to label the molecule of interest with this polymer. A multiple increase in sensitivity could be expected. Although the concept of polymeric fluorescence dyes appears simple, one has to consider stringent requirements for their synthesis and applications. The demands for the dyes, such as quantum yield, Stokes' shift, light stability, etc. match those for monomeric dyes. But the use of polymers with biological compounds bears intrinsic difficulties like low solubility, unspecific interactions, size heterogenity, low yield in covalent attachment, interference with biological activity and so on. In our present work we have synthesized a polymeric dye on the basis of coumarine and attached the polymer to antibodies and streptavidin as model proteins. We report how the problems of heterogeneity could be solved, the yield of conjugation improved and influence on the biological activity minimized. As the result of several examples, we identified unspecific interactions with biological material as most serious difficulties which might be overcome, however, by using another dye but applying the same polymer based concept.

Materials and methods

Chemicals and buffers

All chemicals were of the highest purity commercially available. Buffers were freshly prepared from high purity water (milli-Q-System, Millipore, Neu Isenberg, FRG).

Proteins and antibodies

Streptavidine and Amino-methyl-coumarine-acetic acid (AMCA)-streptavidin was purchased from Boehringer–Mannheim. Streptavidine conjugated to alcaline phosphatase, biotinylated alcaline phosphatase (bio-AP), monoclonal goat-anti-mouse Immunoglobulin G (GAM-IgG) and monoclonal biotinylated rabbit-anti-goat Immunoglobulin G (bio-RAG-IgG) was purchased from Dianova GmbH (Hamburg, FRG).

Coumarine based polymeric dye

The synthesis of the Polymeric Coumarine Dye (PCD) will be described under Results.

Dye-protein coupling

A covalent bond is formed between the carboxyl group of the functional monomer and an amino group of the protein with 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimid) (EDC, Pierce, Munich, FRG) as a catalyst. The reaction is described in detail under Results. Optimized reaction conditions using PCD for conjugation to streptavidin: fractionated dye: $1.8 \cdot 10^{-5}$ M (polymer), streptavidin: $3.7 \cdot 10^{-5}$ M, EDC: 20 mM, 10 mM sodium phosphate pH 7.5 under gentle shaking for 90 min at 25 °C; and for conjugation to antibodies: fractionated dye: $4.7 \cdot 10^{-6}$ M (polymer), antibody: $9.3 \cdot 10^{-6}$ M, EDC: 20 mM, 10 mM sodium phosphate pH 7.5 under gentle shaking for 130 min at 25 °C.

In-vitro transcription

Unbiotinylated and biotinylated (Biotin-21-UTP, Clontech) transcripts from cDNA of viroid-sequence with a length of 73 nucleotides with 13 UTP were synthesized from linearized plasmids with the T7-polymerase transcription system as described elsewhere [10]. For biotinylated transcripts the UTP/biotinylated UTP ratio was 9:1 leading to not more than one biotin group per transcript and thereby minimizing intermolecular crosslinking after addition of streptavidin or dye-streptavidin conjugates. The transcripts were purified with phenol/chloroform and precipitated by ethanol.

Gel filtration of PCD and the products of the coupling reaction

The fractionation of the polymeric fluorescence dye was performed by FPLC-gel filtration chromatography. All chromatographic runs were carried out on a HiLoad 16/60 Superdex 200 prep grade column (Pharmacia, Freiburg, FRG). For quantitative preparations 50 mg PCD in 1ml GF buffer (25 mM Tris, pH 7.2, 0.5 M NaCl, 10% (v/v)

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Ethanol) were applied and eluted from the column with a flow rate of 0.5 ml/min at 0–1 bar pressure. UV-absorbtion at 280 nm or 368 nm (Knauer, Berlin) was recorded and 1 ml fractions were collected (Pharmacia, Freiburg, FRG). The dye of a single fraction was precipitated by three volumes ethanol (96%) at −70 °C.

For preparation of dye–protein conjugates the total coupling reaction, containing 600 – 800 μg of fluorescence dye and various amounts of proteins in 1 ml of GF-buffer was applied and eluted from the column with a flow rate of 2.5 ml/min (pressure and detection unchanged).

Polyacrylamide gel electrophoresis

The fractions from gel filtration of the polymeric dye were analyzed by polyacrylamide (PAA) gel electrophoresis. 10 μl (6 μg) of each sample were added to 10 μl sample buffer containing 20% (v/v) glycerol, 100 mM sodium-acetate buffer pH 5.0 and bromphenol blue. Electrophoresis was carried out using a horizontal 5% PAA (30:0.8 acrylamide: N,N′-methylenbisacrylamide) gel(3 mm × 15 cm × 15 cm) in 50 mM sodium-acetate buffer, pH 5.0, which covers the gel to a depth of 5–10 mm (for 4 h at 200 V and 150 mA). The gel was analyzed on a UV screen at 364 nm.

Agarose gel electrophoresis

PCD-protein coupling products were analyzed by agarose gel electrophoresis. An amount of 10 μl of the sample and 10 μl of sample buffer containing 20% (v/v) glycerol, 100 mM sodium acetate pH 5.0, 100 mM NaCl and bromphenol blue were applied to a horizontal 0.8% agarose gel (7 mm × 10 cm × 7 cm). Electrophoresis was carried out using 50 mM sodium-acetate pH 5.0, 50 mM NaCl, which covers the gel to a depth of 5–10 mm. Constant recirculation of the buffer was required to maintain the pH at 5.0. The gel was analyzed on a UV screen at 364 nm for detecting the fluorescence of the dye. Proteins were transferred by capillary blotting to a nitrocellulose membrane. The assembly for capillary blotting is described elsewhere [11]. A mass flow is accomplished by placing the gel on filter paper, moisted by 10 × SSC-buffer (1.5 M NaCl, 150 mM Na-citrate, pH 7.0) from a reservoir below. The nitrocellulose membrane is placed on the top of the gel, and an absorbent filter paper and paper towels on top of the nitrocellulose membrane. The absorbent paper forces the buffer in 16 h at 25 °C through the gel and the nitrocellulose where the proteins are trapped. The proteins were stained by incubation with biotinylated alcaline phosphatase (bio-AP) for detection of streptavidin, respectively biotinylated rabbit-anti-goat Immunoglobulin G (bio-RAG IgG) and streptavidin conjugated alcaline phosphatase for detection of IgG and Fast-Red-staining [1].

Spectroscopy

Absorption and fluorescence of PCD and of PCD-protein conjugates were analyzed using a Kontron UVIKON 810 spectrophotometer and a Kontron SFM 23 fluorescence spectrophotometer, respectively.

Ultracentrifugation

Sedimentation runs, CsCl density gradient centrifugation and molecular mass centrifugation were carried out in an analytical ultracentrifuge (Spinco model E, Beckman Instruments, Munich). The optical systems used were either the absorption optics equipped with a high-intensity illumination system [12] or a laser-excited fluorescence detection system [13]. For excitation at 364 nm an argon-ion laser (Spectra Physics) was focused to an area of 120 × 120 μm in the center of the cell and is moved radially for scanning. After passing the cell the laser beam is quenched in a light trap of spectroscopic carbon whereas the fluorescence light at 468 nm is collected on the photomultiplier. To reduce background from excitation light two filters (1) edge filter KV 389; 2) interference filter LM 477.8 (both Schott, Mainz)) are used. All runs were performed in a four-hole rotor An-F Ti. Sedimentation runs were carried out with epon single sector center pieces with an optical pathlength of 3.0 mm at speeds between 30000 rpm and 52000 rpm; CsCl density gradient centrifugations with single sector center pieces of an optical pathlength of 3.0 mm or 12 mm at 48000 rpm using a ρ_0 between 1.2 g/cm^3 and 1.6 g/cm^3; sedimentation equilibrium centrifugation with double sector cells of an optical pathlength of 12 mm between 6000 and 12000 rpm. The calculation of the molecular parameters was performed according to the producer's manual [14].

Results

Synthesis of the polymeric fluorescent dye

The procedure for the synthesis of the polymeric fluorescent dye is the result of optimizing the properties of the product with respect to water solubility, fluorescence intensity and accessibility of the functional carboxyl groups for conjugation with the biopolymer.

Synthesis of the functional monomer octaethyleneglycol-methacrylate succinate for conjugation with the biopolymer

In 40 ml of dioxan are dissolved 5 g of commercially available Blemmer PE 350™ (Fig. 1a), 1.13 g Succinanhydrid and 5 mg of 2,6-di-tert-buthylphenole. After addition of five drops of concentrated sulfuric acid the reaction mixture is refluxed for 6 h. The cooled solution is treated with 20 drops of sodium methylat – solution (30% in methanol) to raise the pH to 4, evaporated to dryness and taken up in methylene chloride. After filtration the methylene chloride is evaporated and the residue dried *in vacuo*. The remaining viscous oil contains the functional monomer (Fig. 1b) with 90% purity as judged by ^{1}H-NMR.

Synthesis of the coumarine dye monomer

In 30 ml of dimethylacetamide (DMAC) are dissolved 5 g of 3-(4-amino phenyl)-7 benzenesulfamido coumarine (Fig. 1d) and added 2.56 g of *m*-TMI (isopropenyl-α,α-dimethylbenzyl isocyanate, American Cyanamid, Fig. 1c) dissolved in 10 ml of DMAC. The reaction mixture is heated to 55 °C for 24 h and the course of the reaction checked by IR spectroscopy. The cooled solution is poured in water, the residue filtered, washed successively with acetone and diethyl ether dried *in vacuo*. The dried solid contains the product (Fig. 1e) with > 95% purity as judged by ^{1}H-NMR.

Synthesis of the polymeric coumarine dye

Figure 2 represents the polymeric coumarine dye PCD with its compounds as described above. For the radicalic polymerization in 6 ml of dimethylsulfoxide (DMSO) are dissolved 1.05 g of commercially available sodium styrene sulfonate (Fig. 1f), 0.79 g of the dye monomer (Fig. 1e), 0.79 g of functional monomer (Fig. 1b) and 26 mg of azobisisobutyonitrile. The reaction mixture is heated to 65 °C for 18 h. The cooled solution is precipitated in isopropanol and the filtered residue dried *in vacuo* (yield: 75%).

Size distribution of the polymeric dye

The fractionation of the polymeric dye by gel filtration on analytical scale is shown in Fig. 3. The section (b) of the chromatogramm represents the molecules with sizes in the range of chromatographic resolution of the column. Molecules larger than the pores of the gel elute in the void volume (a). The range of resolved sizes in this peak depends on the salt concentration of the GF-buffer. Higher salt concentrations (up to 0.5 M NaCl) shield the negative charges of the polyanionic PCD and the polymer structure tends to collapse. Thus, even larger molecules, i.e., with a higher number of fluorophors in the polymer, can be fractionated. Addition of 10% (v/v) ethanol reduces hydrophobic interactions of the polymeric dye with the gel medium and leads to better resolution. Peaks (c) and (d)

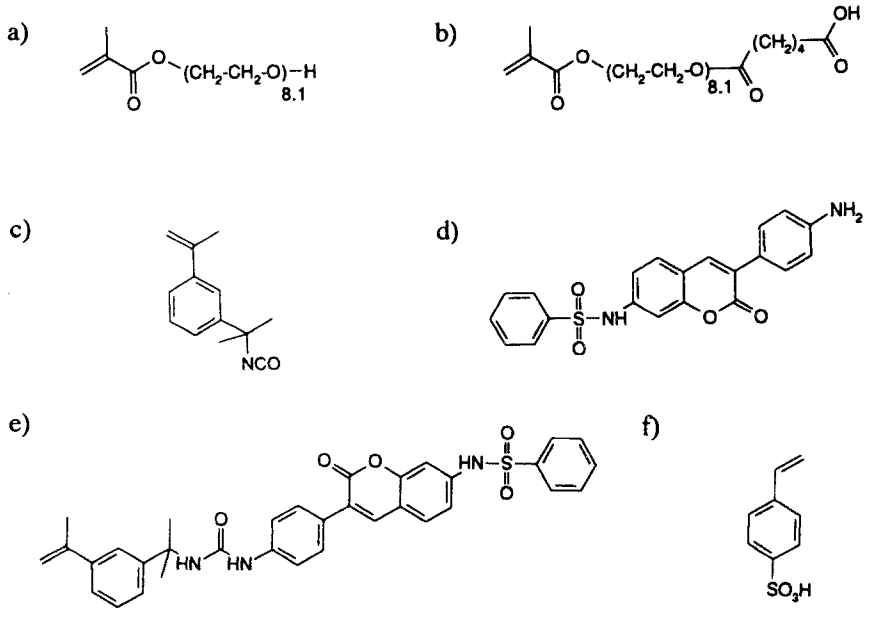


Fig. 1 Components of the polymeric coumarine dye (PCD). Compound a) (Blemmer PE350™) is the precursor of the functional monomer octaethyleneglycolmethacrylate (b). From a coumarine dye (d) and TMI (c) the coumarinemonomer for polymerization (e) is synthesized. Styrene sulfonate (f) represents the monomer responsible for water solubility

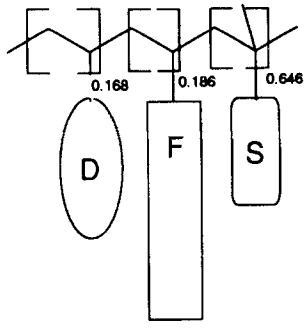


Fig. 2 Polymeric coumarine dye (PCD); D = Coumarine dye (cf.Fig. 1e), F = functional monomer (cf. Fig. 1b), S = sodiumstyrenesulfonate (cf. Fig. 1f); the numbers represent the molar ratios of each monomer in the polymer

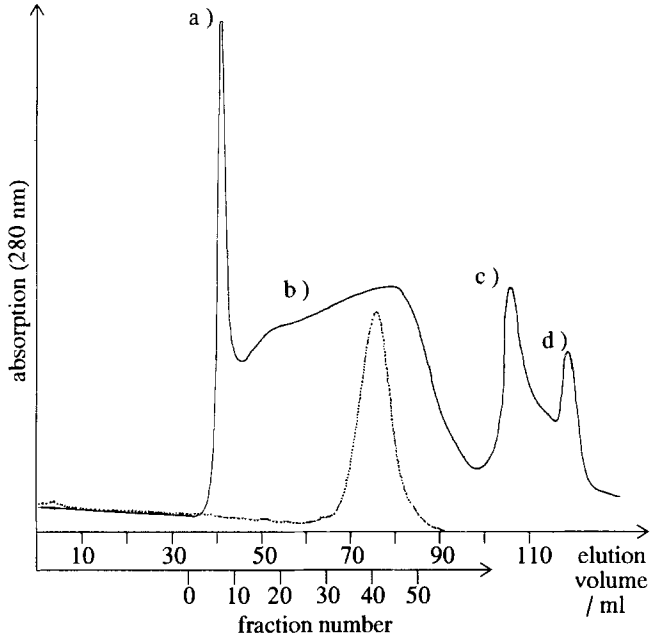


Fig. 3 Analytical gel filtration of PCD (—); rechromatograph of fraction 41 (····); chromatographic conditions: HiLoad 16/60 Superdex 200 prep grade column, 375 μg starting material in 1 ml buffer, 25 mM Tris-HCl, 0.5 M NaCl, 10% (v/v) ethanol, pH 7.2; eluation rate, 2.5 ml/min; detection at 280 nm; for a) b) c) and d) see text

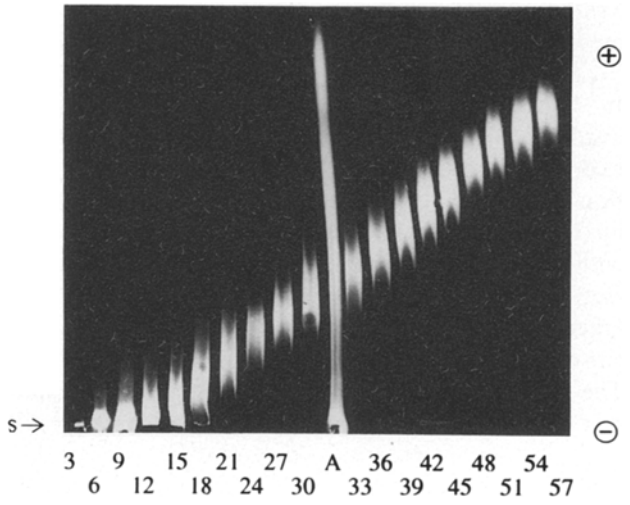


Fig. 4 Analysis of dye fractions from a preparative gel filtration as shown in Fig. 3 by polyacrylamide gel electrophoresis. The numbers of the lanes correspond to the dye fractions (cf. Fig. 3). Lane A shows the unfractionated dye. 10 μg were applied per fraction, Lane A: 45 μg of the unfractionated dye. Electrophoretic conditions:5% polyacrylamide (30:81 acrylamide: N-N' methylenbisacrylamide); buffer: 50 mM Na-acetate pH 5.0; 200 V, 150 mA for 4 h. Fluorescence detection using an UV-screen at 364 nm

represent not polymerized monomers. The coumarine dye monomer interacts with the gel medium by hydrophobic interaction and could only be eluted by washing the column with 25% (v/v) acetonitrile (not shown in the chromatogram). The rechromatogram of a single fraction of the first chromatographic run shows one symmetric peak with a homogeneous size distribution.

The fractions of the gel filtration were analyzed further by polyacrylamide gel electrophoresis (Fig. 4). The polyanionic PCD migrates to the anode and the gelelectrophoretic mobility depends upon the number of charges and the friction in the gel matrix. It is shown in Fig. 4 that the unfractionated dye (lane A) forms a smear through the entire length of the gel, but that single fractions (lane 3–57) form regions of well defined mobility. Smaller dye molecules, which eluted later in gel filtration (Fig. 3), show a higher gel electrophoretic mobility. Fractions of larger molecules, which were eluted in the void volume, do not enter the PAA gel (fractions number 3 and 6 of Fig. 4). From the pattern of the gel electrophoretic mobilities in Fig. 4 it can be concluded clearly that fractions of PCD, which are homogenous in their size according to gel filtration, are also homogenous in the number of charges according to electrophoresis. This is a valuable result of the mechanism of polymerization, as will be discussed later. The fractions of the gel filtration were also characterized by their molecular weights as determined by sedimentation equilibrium runs in the analytical ultracentrifuge. The results are shown in Table 1. For an easy summary of the analysis of the polymeric dyes the dependences of the elution volume and of gel mobility upon the molecular weight are shown in Fig. 5.

Conjugation of polymeric fluorescence dyes with proteins

Strategy

First, we tried to use 6-aminohexanole as the reactive group in a polymeric dye by activating it with mesylchloride under pyridine catalysis, and to react the resulting

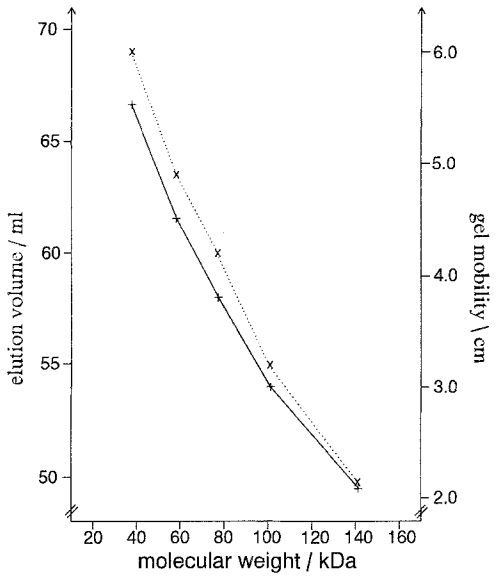


Fig. 5 Dependence of the elution volume (dotted line, data from Fig. 3) and of gel mobility (not interrupted line, data from Fig. 4) upon the molecular weight of the fractionated dye

intermediate (sulfonacidic ester) with a primary aminogroup of the protein. This approach was not successful, since it was shown by detailed analysis that protein and polymeric dye formed complexes only by electrostatic interactions [15]. Because of this failure a polymeric coumarine dye carrying carboxylgroups (PCD) for the reaction with a primary aminogroup of the protein was developed. The reaction is catalyzed by 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimid) (EDC; Fig. 6) which is well known for its ability to conjugate small molecules ($M_r < 500$) carrying carboxylgroups to primary aminogroups of proteins [16, 17].

According to the literature [18], the coupling reaction consists of two steps (Fig. 6). In a nucleophilic addition reaction an oxygene of a carboxylgroup of the dye interacts with the carbon of the carbodiimide group of the EDC-molecule. In the second reaction step the nitrogen of one aminogroup of the protein reacts via a nucleophilic substitution reaction with the intermediate product. In aqueous solution the two reactions form a peptide bond between a carboxylgroup of the dye and a primary aminogroup of the protein. The EDC molecule leaves the reaction as an urea derivative.

Analysis and optimization of the coupling reaction by agarose gel electrophoresis

A gel electrophoretic system was developed particularly to analyze and optimize the yield of coupling of polymeric dyes to poteins. At pH > 2 – 3 PCD is a polyanion. Both proteins (streptavidin and IgG), which were tested for coupling, have pI-values around 7 and are positively charged at lower pH. Thus the opposite charges at slightly acidic pH of dye and proteins were used for a gel electrophoretic analysis. In Fig. 7 such an analysis is shown, on the left side the original experiment and on the right side as a schematic drawing for the readers convenience. Both, dye and protein, were made visible independently after a capillary transfer of the compounds to a nitrocellulose membrane, the dye due to its own absorption or fluorescence and the protein after an immunostaining with the Fast-Red reaction.

PCD migrates to the anode (lane 1), whereas the protein (lane 2, in this case streptavidin) migrates to the cathode. Due to the high charge of the dye, conjugates of the dye and proteins (lane 3) are charged negatively, but less than the uncoupled dye. This effect and the higher friction of the dye-protein-conjugates result in a retarded band. The result in Fig. 7 demonstrates that this type of electrophoresis is a simple and fast procedure to test for coupling. The coupling reaction could be analyzed systematically by varying PCD-protein ratios. The analysis was applied to streptavidin (Fig. 8) and antibodies (data not shown) as the protein component. The molar streptavidin-PCD ratio was varied from 1.4:1 to 4.3:1. PCD fraction 20 (cf. Fig. 3) with 170 kDa was used for coupling. In Fig. 8A the fluorescence of the dye moiety was depicted directly, in Fig. 8B the streptavidin moiety was stained

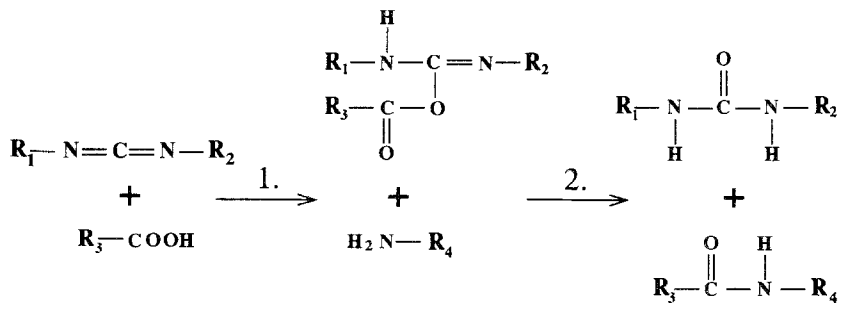


Fig. 6 Scheme of the EDC-mediated coupling reaction [18]. First step: EDC with R_1 (ethyl group) and R_2 (dimethyl-aminopropyl group) reacts with a carboxyl group of the polymeric dye (R_3-COOH). Second step: The activated carboxyl group reacts with a primary aminogroup of the protein (R_4-NH_2)

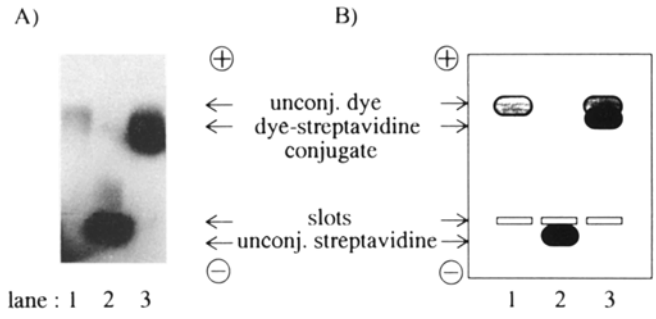
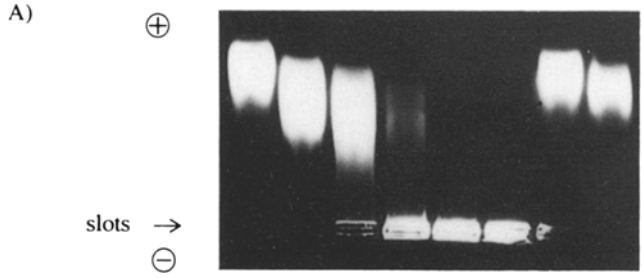
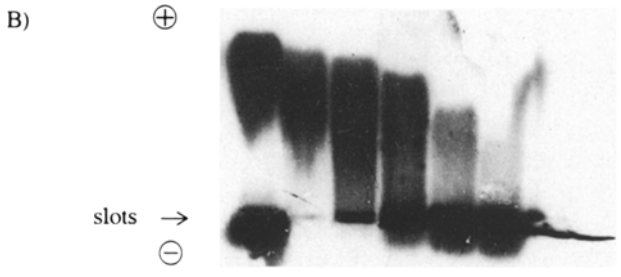


Fig. 7 Electrophoretic analysis of dye-protein conjugate, unconjugated dye, and unconjugated protein. After electrophoresis the compounds are transferred to a nitrocellulose membrane by capillary transfer. The dye moiety is detected by its yellow color, the protein (streptavidin) by staining with "fast-red" (cf. Materials and Methods). In panel A a black and white photograph is shown, in panel B a schematic drawing of the location of the different compounds. Lane 1:10 μg polymeric coumarine dye PCD fraction 32 (yellow), lane 2:5 μg streptavidin (red), lane 3:10 μg PCD fraction 32 conjugated with 5 μg streptavidin under catalysis of 50 mM EDC. Electrophoretic conditions: 0.8% agarose, 100 mM Na-acetate, 50 mM NaCl, pH 5.0; 50 V, 200 mA and 90 min

Fig. 8 Electrophoretic analysis of the yield of conjugation of PCD and streptavidin in dependence upon the streptavidin concentration. Electrophoretical conditions: 0.8% agarose; buffer: 50 mM Na-acetate, pH 5.0, additional 50 mM NaCl in the application and gel buffer; 50 V, 100 mA for 2 h. A) PCD recorded by fluorescence, 10 μg PCD per lane. B) Streptavidine recorded after capillary transfer on nitrocellulose and fast-red-staining. The ratio of the conjugation educts is listed in the table. $^{+=}$ addition of EDC, $^{-=}$ no addition of the corresponding educt



lane	1	2	3	4	5	6	7	8
molar ratio of PCD	1	1	1	1	1	1	1	1
molar ratio of strept.	1.4	1.4	2.1	2.9	3.6	4.3	-	-
EDC (120 mM)	-	+	+	+	+	+	-	+



after transfer to a membrane as described for the experiment of Fig. 7. As a control lane 1 in panel A shows the dye incubated with streptavidin but without EDC. The dye migrates with the same mobility as compared to the case when incubated without streptavidin (lane 7, panel A). A major portion of streptavidin (lane 1, panel B) is not conjugated and migrates to the anode, the other portion is bound electrostatically to the dye and moves to the cathode. These electrostatic complexes are clearly different from the covalent conjugates obtained after incubation in the presence of EDC. Firstly, the analysis in lane 2 demonstrates that the dye is now retarded because of the covalent type of complex (panel A) and secondly, that the streptavidin is 100% migrating in the complex with the dye (panel B). One should note that the presence of EDC alone, i.e., in the absence of streptavidin, affects the migration of the dye, as can be seen from a comparison of lanes 7 and 8 in panel A. The unwanted electrostatic complex could be reduced to a minimum for gel electrophoretic analysis of PCD-antibody conjugations by addition of 50 mM NaCl to all buffers (data not shown). Because streptavidin is sensitive against oxidation [19] by the chlorine gas developing during electrophoresis, NaCl is added only to the application and gel buffer, but not to the electrophoresis buffer. The retardation of the dye band due to coupling with streptavidin was enhanced by increasing amount of streptavidin (lane 3, panel A). With further increasing streptavidin concentration a large net-work of dyes and streptavidin links are formed due to the multivalent coupling activity of both compounds 1 (lane 4 to 6, panel A); these networks do not enter the agarose gel. Furthermore, an increasing portion of uncoupled streptavidin can be seen in panel B (lane 4 to 6).

For PCD-antibody conjugations the efficiency of the coupling reaction could be increased to approximately 100% of the dye (data not shown).

Preparative separation of PCD-protein conjugates from uncomplexed compounds by gel filtration

The products and the educts of the coupling reaction were seperated by gel filtration. To all buffers 0.5 M NaCl was added to prevent electrostatic interactions between proteins and dye as mentioned above (see the previous section). An amount of 10% ethanol was added to reduce hydrophobic interactions between PCD and the gel matrix of the column. In Fig. 9 chromatograms of a time-course of a coupling reaction, using the 38 kDa PCD fraction and a molar PCD-streptavidin ratio of 2.1:1 are shown.

After 0, 45, 90, and 120 min reaction time the mixture was injected onto the column. Uncoupled streptavidin (d) elutes at 84 ml after injection, the uncoupled PCD (c) at

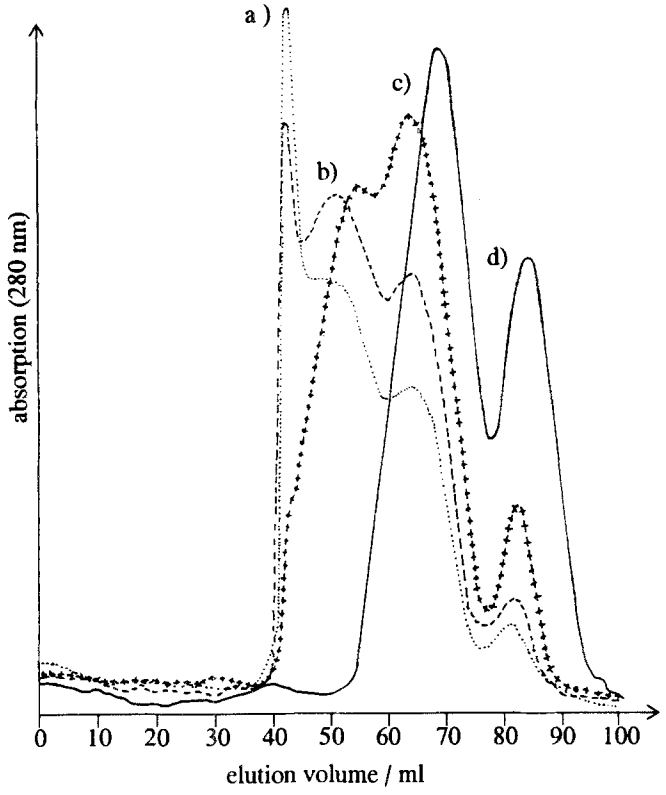


Fig. 9 Gel filtration analysis of the yield of conjugtion of PCD and streptavidin in dependence upon the reaction time. Analysis after 0 (not interrupted line), 45 min (crossed line), 90 min (marked with little lines) and 135 min (dotted line); chromatographic conditions: 10 μg of PCD and 33 μg streptavidin in 12 μl; column: HiLoad 16/60 Superdex 200 prep grade; eluent: 25 mM Tris, 0.5 M NaCl, 10% (v/v) ethanol, pH 7.2; flow rate: 2.5 ml/min; pressure: 0–1 bar; for a), b), c) and d) see text

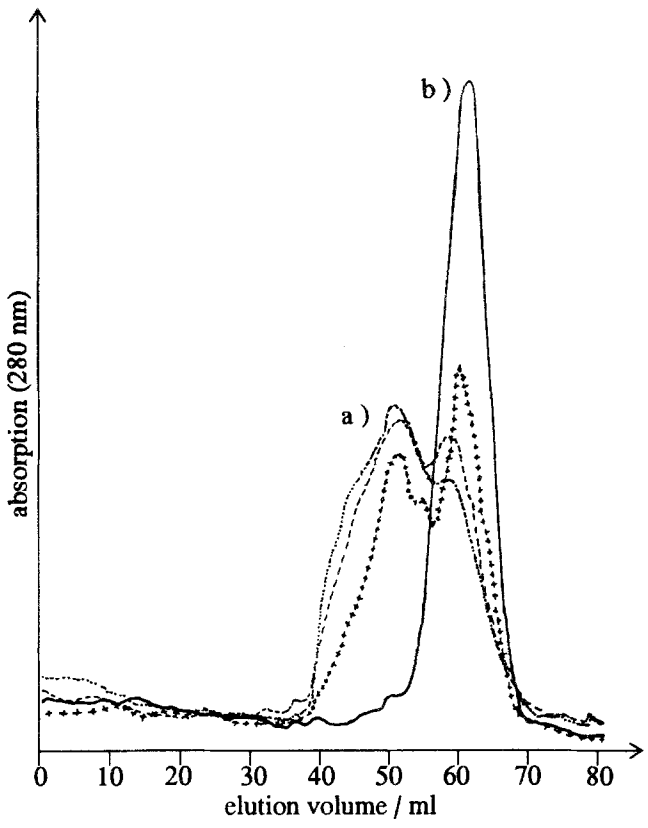


Fig. 10 Gel filtration analysis of the yield of conjugation of PCD and antibodies in dependence upon the reaction time. Analysis after 0 (not interrupted line), 40 min (crossed line), 85 min (marked with little lines) and 135 min (dotted line); chromatographic conditions: 10 μg of PCD (fraction 29) and 32.4 μg antibodies; sample volume: 20 μl; column: HiLoad 16/60 Superdex 200 prep grade; eluent: 25 mM Tris, 0.5 M NaCl, 10% (v/v) ethanol, pH 7.2; flow rate: 2.5 ml/min; pressure: 0 - 1 bar; for a), b), c) and d) see text

64 ml; the PCD-streptavidin conjugate (b) at 50 ml. With increasing reaction times (0, 45, 90, 120 min in Fig. 9) the concentrations of the uncoupled components decrease, those of the conjugate increase. Network-like conjugates (a) comprising several PCD and streptavidin molecules (see the previous section) are formed at reaction times longer than 45 min and elute with the void volume. At a reaction time of 90 min preparative separations of PCD-streptavidin conjugate were optimal, since the concentration of the uncoupled PCD is already low and the amount of the network-like conjugates is still low.

The corresponding separations of antibody-dye conjugates using 100 kDa PCD fraction and a molar PCD-antibody ratio of 2:1 are shown in Fig. 10.

After zero reaction time the PCD and the antibodies elute in a common peak (b) at 62 ml. With increasing reaction time the peak of the free components decreases and the new peak at 50 ml elution volume represents the PCD-antibody conjugate (a). In contrast to the PCD-streptavidin conjugate, no network-like conjugates were formed. Therefore, the reaction time for preparative separations could be extended to 135 min, which results in a higher yield of conjugate formation.

Characterization of the PCD-protein conjugates by analytical ultracentrifugation

The hydrodynamic characterization of PCD and the PCD-protein conjugates was performed by sedimentation velocity runs in the analytical ultracentrifuge with fluorescence detection system (Fig. 11).

Figures 11 A and B show sedimentation profiles of two different fractions of the uncoupled PCD. The narrow boundaries confirm the homogeneity of the PCD fractions as concluded before from the rechromatography and the polyacrylamide gel electrophoresis. The sedimentation coefficients are listed together with the M_r-values in Table 1. In Fig. 11C and 11D the sedimetation profiles of the

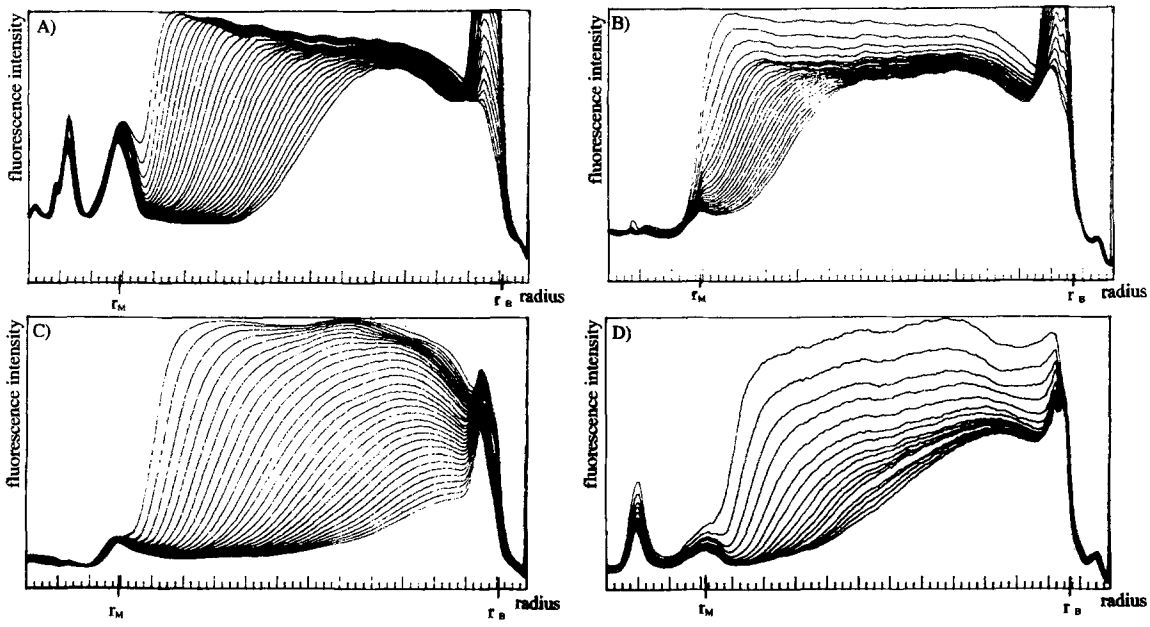


Fig. 11 Sedimentation profiles of PCD fraction 22 (A), fraction 29 (B), the corresponding PCD (fraction 22)- streptavidin conjugate (C), and the corresponding PCD (fraction 29)- antibody conjugate recorded with the fluorescence detection system at 364 nm. sample: 5 ng (A), 4 ng (B), 12 ng (C) and 10 ng (D); buffer: 20 mM Na-phosphate, 0.5 M NaCl, pH 7.5; cell volume: 80 μl; optical path-length: 3 mm; rotor speed: 40.000 rpm (A and C), 32.000 rpm (B and D); r_M = radius of the meniscus, r_B = radius of the bottom; differences between two consecutive scans are 4 min (A and C), 6 min (B and D)

PCD-protein conjugates as eluted from the peak fraction of the chromatography (Fig. 9 and 10, respectively) are shown. For conjugation with streptavidin the PCD fraction 22 (Fig. 3) and with antibodies PCD fraction 29 were chosen. To prevent electrostatic interactions between dye and proteins 0.5 M NaCl was added, thus only covalently linked conjugates were detected. The drastic increase of the sedimentation coefficient as recorded on the dye fluorescence, if compared to those of the free PCD (Figs. 11A and B), demonstrates formation of the conjugates. From the shape of boundaries of the conjugates, one might conclude that the major portion of the streptavidin-dye conjugates represents a uniform stochiometry, whereas the broad boundary of the antibody-dye conjugates indicates more than one type of conjugates. The finding that the sedimentation coefficient exceeds the sum of that of the free compounds shows that either more than one of the educts are in the conjugate or at least one of the compounds, most probably the PCD moiety, undergoes a drastic conformational change during conjugation. This effect will be outlined in the discussion. In Table 1 the sedimentation coefficients of all samples are summarized.

The molecular weights of single PCD fractions were determined with the analytical ultracentrifuge, those of streptavidin and IgG were taken from literature. For fluorescence dye-protein conjugations the molecular weights of the single components were added. Two possibilities, i.e., one PCD molecule conjugated with one protein ($n = 1$) and one PCD molecule conjugated with two protein molecules ($n = 2$) were assumed to calculate the molecular weight of the conjugate. Friction coefficients were derived from the Svedberg equation (Eq. (1)). The partial specific volume of PCD ($\bar{v}_{PCD}$) was 0.649 cm^3/g, of antibodies ($\bar{v}_{AB}$) 0.739 cm^3/g [20] and of streptavidin ($\bar{v}_{SA}$) a value of 0.77 cm^3/g as for typical proteins [20] was assumed, leading to the specific volume of the PCD-protein conjugates (v_C) (Eq. (2)). The Perrin-factors (P) represent the ratio of the experimental friction coefficients (f) and the value of a sphere ($f_{sph.}$) with the same molecular weight (Eq. (3)).

$$S = \frac{m}{f} \cdot (1 - \bar{v} \cdot \rho_L) \tag{1}$$

$$\bar{v}_C = \frac{m_{PCD} \cdot \bar{v}_{PCD} + n \cdot m_{SA/AB} \cdot \bar{v}_{SA/AB}}{m_{PCD} + n \cdot m_{SA/AB}} \tag{2}$$

$$P = \frac{f}{f_{sph}} \tag{3}$$

S = Svedberg unit, m = mass (m_x = mass of component X), $\bar{v}$ = partial specific volume, ρ_L = density of the solution, for other shortenings see text.

Table 1 Summary of the hydrodynamic properties of the compounds used for the covalent conjugation and their products

	Unconjugated PCD						Unconjugated proteins		PCD protein conjugates, molar ratio of			
	fract. 18	fract. 22	fract. 23	fract. 29	fract. 34	fract. 38	fract. 44	strepta-vidin	IgG	PCD and streptavidin		PCD and anti-bodies	
										1:1	1:2	1:1	1:2
Molecular weight/kDa	(200)*	(150)*	140	100	76	58	38	60	150	210	270	250	400
Sedimentation value/S	6.8	5.7	–	4.3	–	–	2.8	4.0	6.8	11.5	11.5	12.3	12.3
Friction coefficient/ $[g \cdot s^{-1}]$	$1.7 \cdot 10^{-7}$	$1.5 \cdot 10^{-7}$	–	$1.3 \cdot 10^{-7}$	–	–	$7.8 \cdot 10^{-8}$	$6.6 \cdot 10^{-8}$	$9.6 \cdot 10^{-8}$	$9.8 \cdot 10^{-8}$	$1.2 \cdot 10^{-7}$	$9.9 \cdot 10^{-8}$	$1.5 \cdot 10^{-7}$
Perrin factor	2.1	2.0	–	2.1	–	–	1.7	1.16	1.24	1.18	1.32	1.17	1.45

* extrapolated data according to Fig. 5

Optical properties of PCD and its conjugates with streptavidin

Table 2 summarizes the optical properties of the coumarine dye monomer, of PCD and its conjugates with streptavidin. Two fractions of PCD are included in the comparison. All samples show the same excitation- and emission maxima and consequently the same Stooks' shift. The quantum yield of the polymeric dye is reduced to 57%–67% compared to its monomer. The molar extinction coefficients, if based on dry weight and molecular weights are 60-fold higher for the polymer from fraction 22 as compared with the monomeric dye and 13-fold for fraction 44, respectively. These extinction coefficients are in good accordance with those expected from the numbers of fluorophors in the polymer. With regard to the quantum yield the increase of the fluorescence intensity per molecule is about 40-fold for the polymer of fraction 22 and 9-fold for fraction 44 as compared to the monomer.

The extinction coefficients were calculated at 368 nm using solutions with defined concentrations. The relative quantum yield was obtained by comparing the fluorescence signals of the polymeric dye fractions and its streptavidin conjugates with the monomer. The quantum yield of the monomer was set to 1.0.

Biological activity of the streptavidin in the dye-protein conjugate

The biological activity of streptavidin is its capability to bind biotin with very high affinity. If biotin is linked covalently to a biological molecule of interest, for example a nucleic acid, the affinity to streptavidin is utilized to record the streptavidin-dye conjugate and to locate hereby the nucleic acid. Therefore, we tested the biotin binding activity of streptavidin if conjugated with PCD. Since the sedimentation coefficients of the compounds are too similar for a clear separation and electrostatic interactions cannot be ruled out completely in gel electrophoresis, we have chosen CsCl density gradient centrifugation to analyze the streptavidin binding of biotinylated nucleic acids. In this method macromolecules are fractionated according to their buoyant density. Since those values are 1.535 g/cm^3 for PCD, 1.43 g/cm^3 for the PCD-streptavidin conjugate and > 1.9 g/cm^3 for the biotinylated RNA, the free compounds and the complex formed via biotin-streptavidin binding should be separable. As a model system for nucleic acids a biotin-labeled *in vitro* RNA transcript (73 nucleotides) was used.

Figures 12 A and B show the results of density gradient centrifugations of mixtures of the PCD-streptavidin conjugate and non-biotinylated RNA (panel A) and biotinylated

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Table 2 Optical properties of single fractions of PCD and the corresponding PCD-streptavidin conjugates as compared with the coumarine dye monomer

	Coumarine dye monomer	PCD fraction 22	PCD (fraction 232) -streptavidin conjugate	PCD fraction 44	PCD (fraction 22) -streptavidin conjugate
Molecular weight/Da	438.5	150.000	210.000	38.000	98.000
Extinction coefficient ε_{368nm} [$M^{-1} \cdot cm^{-1}$]	$2 \cdot 10^4$	$1.2 \cdot 10^6$	$1.2 \cdot 10^6$	$2.6 \cdot 10^5$	$2.6 \cdot 10^5$
Maximum of excitation/nm	368	368	368	368	368
Maximum of emission/nm	472	472	472	472	472
Relative fluorescence quantum yield	1	0.67	0.65	0.67	0.57
Number of coumarines per polymer ($n = \varepsilon_{368nm}$ [polymer]/ ε_{368nm} [monomer])	1	60	60	13	13
Increase of sensitivity ($n \cdot$ relative fluorescence quantum yield)	×1	×40	×38	×9	×8

RNA (panel B), respectively. The density of the solution before centrifugation was 1.450 g/cm^3 (A) and 1.451 g/cm^3 (B), respectively. Only one band with a buoyant density of 1.43 g/cm^3 was detectable in the gradient of panel A, which represents uncomplexed PCD-streptavidin conjugate. It is obvious that the fluorescence compound is shifted to higher density due to its binding to the biotinylated RNA (panel B), and the shift is so large that the peak is not resolved from the bottom position of the density gradient in panel B. If a density of 1.517 g/cm^3 was chosen before centrifugation (panel C) a clear band of the PCD-streptavidin-RNA complex could be resolved at 1.54 g/cm^3. An increase of the buoyant density of 0.11 g/cm^3 for the PCD-streptavidin conjugate was measured due to complex formation with biotinylated RNA. This analysis has demonstrated that streptavidin did not loose its biotin-binding activity after conjugation with PCD and that the affinity is sufficiently high to form a stable complex even in 4.5 M CsCl.

Unspecific interactions of PCD with membranes and other cellular compounds

As mentioned before a typical application of fluorescence dyes in biology is the analysis of location and concentration of a specific nucleic acid (scheme in Fig. 13A). For this purpose an amount of a nucleic acid (for testing RNA-trancripts) is transferred to a blotting membrane. A complementary, biotin-labelled RNA is hybridized to the membrane-bound RNA, and then the PCD-streptavidin conjugate is bound to the biotin-groups in a consecutive incubation. The resulting fluorescence signal can be taken to localize the nucleic acid or to estimate its concentration respectively. When those experiments were carried out with the conjugated PCD and different membranes (nylon- as well as nitrocellulose-membranes were used) specific signals were obtained only for high concentrations of the nucleic acid of interest (20 ng RNA-transcript, data not shown). Directly blotted PCD (scheme in Fig. 13B) could be detected in a minimum amount of 5 ng (data not shown). Both directly blotted PCD and PCD-streptavidin conjugates after incubation to biotinylated nucleic acids exhibited a high unspecific background, which obscured any lower specific signal. Most probably hydrophobic interactions of the coumarine in the polymer, which were detected also during chromatography (see earlier), are responsible for these unspecific interactions.

When a biotinylated DNA-probe was applied in *in situ* hybridizations in order to detect specific sequences in chromosomes from *Drosophila melanogaster*, labeling with the PCD-streptavidin conjugate gave similar unspecific results. The whole chromosomal material was stained with the PCD (data not shown) so that a specific signal could not be detected. The consequences of these unsuccessful applications to biological questions will be discussed below.

Discussion

Charge and size distribution of the polymeric coumarine dye (PCD) as analyzed by ultracentrifugation, gel filtration and electrophoresis

As a product of radical polymerization the polymeric coumarine dye (PCD) was obtained with a wide distribution of molecular weights and charges. The mixture could

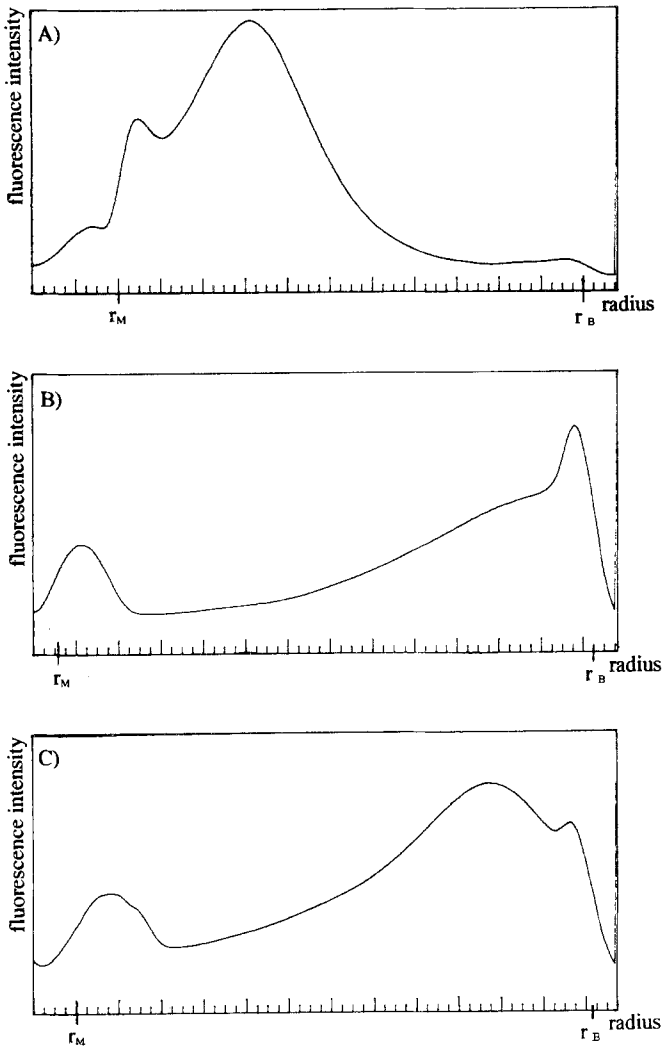

Fig. 12 CsCl density gradient centrifugation of PCD (fraction 22)-streptavidin conjugate incubated with unbiotinylated- (A) and biotinylated RNA (B and C), recorded with fluorescence detection system at 364 nm. ρ_0: 1.450 g/cm^3 (A), 1.451 g/cm^3 (B) and 1.517 g/cm^3 (C); buffer: 100 mM Na-phosphate pH 7.5; cell volume: 80 μl; optical pathlength: 3 mm; rotor speed: 48.000 rpm; r_M = radius of the meniscus, r_B = radius of the bottom

be fractionated by gel filtration on a preparative scale. In gel filtration on a dextran-based column in aqueous solution adsorptive interactions could be avoided, whereas anion-exchange chromatography was not successful because of unspecific interactions (data not shown). Since PCD bears some similarity to a nucleic acid in respect to size, water solubility and polyanion characteristics, we applied polyacrylamide gel electrophoresis, which is the standard method for nucleic acid analysis but quite unusual for the analysis of synthetic polymers. This type of analysis was indeed quite successful with PCD and demonstrated (Fig. 5) that the gel electrophoretic mobility and hydrodynamic volume in gel filtration depend upon the molecular weight in a very similar manner. From this relationship it could be demonstrated that the fraction of charged monomer incorporation remains nearly constant over the whole polymerization process.

Conjugation of the polymeric dye to proteins

Fractionated PCD was used for the conjugation to proteins. A peptide bond between the carboxylic group of a functional subunit of PCD and a primary amino group of the protein is mediated by EDC. The existence of a covalent bond could be verified by gel electrophoresis, gel filtration and analytical ultracentrifugation. Also for this type of analysis gel electrophoresis proved to be a very useful analytical tool for synthetic polymers. It was essential to carry out the analyses in the presence of 50 mM NaCl during gel electrophoresis and 0.5 M NaCl during gel filtration, respectively, in order to rule out non-covalent complexes formed by electrostatic interactions.

Structure of PCD and its conjugates with proteins

Form parameters of the fractionated PCD and its protein conjugates were determined by analytical ultracentrifuga-

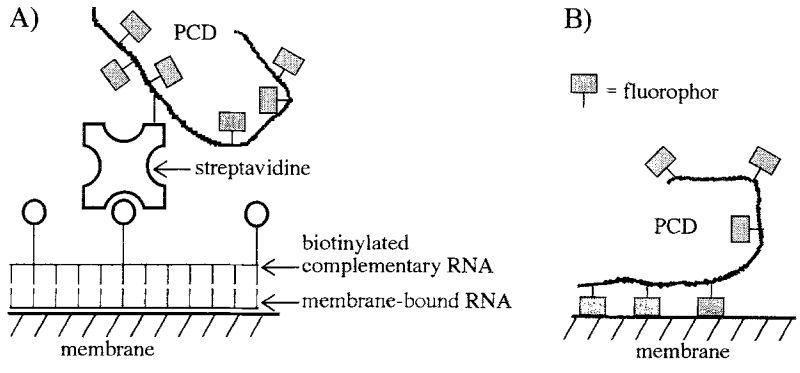

Fig. 13 Scheme of fluorescence detection of PCD after blotting on a transfer membrane (panel B) and of blotted RNA-samples after hybridization and incubation with PCD (panel A)

tion. Perrin factors of 2 and 2.1 were obtained for PCD-fractions 22 and 29, respectively, showing that the polymer structure definitely does not collapse to a sphere-like shape. An extended structure is in accordance with the behavior on gel filtration. Perrin factors of 1.2 were calculated for PCD-streptavidin and for PCD-antibody conjugates, if 1:1 conjugates were assumed. The factors would be 1.3 and 1.5 for the streptavidin and antibody conjugates, respectively, if two proteins were linked to one PCD. For both possibilities, however, the data show that the PCD-protein conjugates assume a more collapsed structure than the unlinked PCD. One might assume that the polymeric dye is wrapped at least partially around the protein.

Prospects

PCD (fraction 22) showed a 60-fold larger molar extinction coefficient as compared with the coumarine dye monomer. Because of the somewhat lower quantum yield the fluorescence intensity is 40-fold higher. Compared with the commercially available amino-methyl-coumarin-acetate-streptavidin (AMCA-streptavidin, Boehringer-Mannheim) with 6-8 coumarine derivates per streptavidin PCD is more sensitive by a factor of 4. Although the advantage of PCD does not appear very large, it might be raised by applying larger polymers and a dye subunit with less tendency for quenching. Coumarine had the favorably large Stokes shift but its extinction coefficient does not appear very favorable if it is compared to the four-fold higher extinction coefficient of the dye fluorescein. The biggest disadvantage of PCD, however, is the tendency for unspecific, mostly hydrophobic interactions. This not only narrowed down the possibilities for fractionation due to the unspecific interactions with most chromatographic matrices, but also and most significantly prevented biological applications with high sensitivity. Coumarine-based polymeric dyes stick unspecifically to test membranes and biological tissue. We could estimate roughly from the data in Fig. 13 that the sensitivity of nucleic acid detection might be increased by a factor of 1000 if unspecific background from dye-membrane interaction could be avoided. This type of summary, although unfavorable for PCD, does not mean that one should not follow up the approach described in this work. In fact, the main progress of the present work are the methodological developments for synthesizing, preparing, characterizing, and working out applicability test for polymeric dyes. Since we know that fluorescein exhibits the better optical data (except Stoke's shift) and that preliminary data showed much less unspecific interactions, it appears straight forward and most promising to utilize the methods of the present work and develop a fluorescein-based polymeric dye.

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