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The influence of labelling mechanisms on the fluorescence behaviour of polymers bearing fluorescein labels

Mel Carvell[a], Ian D. Robb[b,*] and Phil W. Small[c]
[a]*Unilever Research Laboratory Port Sunlight, Merseyside L63 3JW UK*
[b]*Centre for Water Soluble Polymers; North East Wales Institute, Mold Rd, Wrexham LL11 2AW UK*
[c]*32 Barrymore Way, Bromborough. Merseyside L63 0HN UK*
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The labelling of macromolecules by fluorescent labels is a well established method for studying their motion, location and environment. There are a wide range of labels and methods for attaching these to polymers, and the resulting fluorescence behaviour can depend on both the label and labelling method. Fluorescein is a commonly used label because of its relative ecological safety and its pH dependence. Various methods for attaching fluorescein (via its amine derivative) and eosin to water soluble polymers were investigated, together with the effect on fluorescence behaviour. For fluoresceinamine, it was necessary to prevent the lone pair on the nitrogen from quenching the fluorescence by using an amide (via Woodwards reagent) or isothiocyanate as the linking group between the label and macromolecule. This also largely preserved the well known pH dependence of the fluorescence of fluorescein. Eosin (tetra bromo fluorescein) provides a constant fluorescent intensity over a wider pH range than fluorescein and can be linked to macromolecules via the carboxyl group without significantly influencing this pH stability. © 1997 Elsevier Science Ltd.

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INTRODUCTION

The behaviour of macromolecules in solution can be studied by attaching labels of various types, and inferring motion or location of the polymer from the label. Fluorescent labels have often been employed for this, using the spectral shape, position or depolarisation of light to obtain information about the macromolecule. A number of reviews[1–3] have described these applications, the major use in aqueous systems being in biological molecules.

There are a number of labels to chose from, having various lifetimes, susceptibilities to quenching and groups in the molecule to facilitate attachment to a macromolecule. It is often the preferred route to incorporate the label into the macromolecule during its preparation, though, as with many biopolymers such as cellulose, this is not always possible. In these cases it may be necessary to attach the label to an existing polymer, which can involve a careful choice of label and linking mechanism. In our studies on labelled polymers we preferred labels that had a low toxity in biological systems to minimise any problems that could arise if the label were to detach during experiments. From this point of view, fluorescein and its derivatives are suitable labels as they are relatively non-toxic, have a high quantum efficiency and can be pH sensitive[4].

The purpose of this study was to examine various routes for attaching fluorescein and related derivatives to polymer chains, and the influence of these different labelling mechanisms on the spectra. A versatile form of fluorescein is fluorescein amine, the amine group providing a convenient site for attachment to macromolecules. Munkholm et al.[5] have shown that quenching of fluorescein amine is strongly influenced by the lone pair of electrons on the nitrogen of the amine. However, if the nitrogen is converted to an amide or secondary nitrogen, the fluorescence is largely restored. Thus, the bond between the label and polymer is likely to have a significant effect on the spectra obtained; it was the intention of this work to establish the effect of these linking mechanisms, especially for fluorescein amine, on the fluorescence spectra.

Whilst the pH dependence of the fluorescence of fluorescein can be an advantage to probe pH, it can be a disadvantage where pH independence, e.g. for quantitative analysis, is sought. The fluorescence of the tetra bromo derivative of fluorescein, eosin-Y, is less pH dependent and this was also studied as a possible label.

EXPERIMENTAL

The labels used, fluorescein (Fl), fluorescein amine, isomer 1 (Fla), Eosin-Y (EoY) and Ethyl eosin (EtEo) were obtained from Aldrich and used as received. For EtEo, the ethyl group is connected via the phenyl-carboxyl group. Poly(acrylic acid), (PAA), molecular mass 230 000 was obtained from BDH. The sodium carboxymethyl cellulose (SCMC) was obtained from Hercules and had a degree of substitution of 0.7 and viscosity average molecular mass of 250 000. The poly(styrene sulphonate-maleic anhydride) (PSS-MA) which had a styrene/maleic anhydride mole ratio of 2.0 and a molecular mass of 35 000, was obtained from National Starch. The labelled polymers were prepared in the following way.

* To whom correspondence should be addressed

Table 1 Bond type linking label and polymer

Polymer	PAA, PSS-MA	SCMC	Dextran
Bond type	Amide	Secondary amine	Isothiocyanate

Table 2 Number of monomer units/label (NI)

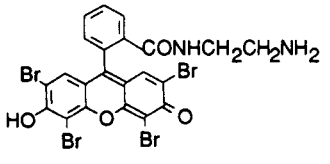
Polymer	PAA	PSS-MA	SCMC	Dextran
NI	160	5,200	260	140

The PAA and the PSS-MA were labelled by the well-known route[6] using Woodwards' reagent K (N-ethyl-5-phenyl iso oxazolium-3'-sulphonate), i.e. the polymer at pH 10 was reacted with Woodward's reagent K (one equivalent for each monomer unit) and allowed to stand overnight. Fla (0.5 equivalents) was added to the mixture and allowed to stand at room temperature for 5 h. The polymers were collected and separated from the unreacted label by repeated precipitation with acetone followed by dialysis. A second labelling route was used for SCMC; this was to dissolve the Fla (1 equivalent) in a minimum amount of water and add cyanuric chloride (1 equivalent) in dioxane at room temperature. After 30 min reaction at 50°C, pH 10, this solution was added to the polymer solution (a few percent in water; 3 equivalents of monomer per equivalent of cyanuric chloride) and kept at 80°C for 3 h. The polymer was again purified by repeated precipitation with acetone. Fluorescein attached to dextran (molecular mass 9000) using the 4,5'-dimethyl fluorescein isothiocyanate was obtained from Sigma. Eosin labelled dextran, molecular mass 71 000 was obtained from Aldrich. Thus, the bonds linking the labels to the polymers vary and are listed in *Table 1*.

An alternative method for labelling PAA using Eo was also used. In this method the Eo was reacted with ethylene diamine in dimethyl formamide, to produce the structure shown in *Scheme 1*. This was subsequently linked to the PAA via the Woodwards reagent K method. The labelled polymer was recovered by precipitation, excess label removed by dialysis and the polymer freeze dried.

Absorbance measurements were made on a Perkin Elmer 5 UV/Vis spectrophotometer and emission measurements were made on a Perkin Elmer 3000 fluorimeter using an excitation wavelength of 488 nm. Solutions were made up to concentrations of fluorescent label of $0.5–1.0 \times 10^{-6}$ mol dm^{-3}. Labelling densities (*Table 2*) on the polymers were calculated by comparing the absorbance of Fla and the labelled polymer at pH 10. At this pH it was assumed that the extinction coefficients of the label free in solution and on the polymer were the same. The concentration of polymer in each system was about 10^{-3}%, well below C^*, the overlap concentration.

Eosin-$NHCH_2CH_2NH_2$ =



Ethylene diamine attached to eosin.

RESULTS

The extinction coefficients for Fla and the polymer conjugates are shown in *Figure 1*. Those quoted for the labelled polymers are not absolute as they were calculated in the following way. The absorbance of the Fla free in solution was assumed to be equal to that when attached to the polymer—reasonable for absorbance, but not for emission. From the known weight of polymer used, the labelling density was calculated. The pH dependence of the extinction coefficients was calculated using these densities. Furthermore, the coefficients refer to the maximum in the absorption band, which changes with pH as shown in *Figure 3*. No corrections or normalisations were attempted for the small errors arising in weighing procedures and intensity measurements in *Figure 1*. The coefficients for Fla and the low charge density polymers are quite similar, whilst those of higher charge density, PAA and PSS-MA, show a reduction at intermediate pH (≈7). The Fla conjugate may be influenced not only by bonds connecting the label to the polymer, but also by the environment of the

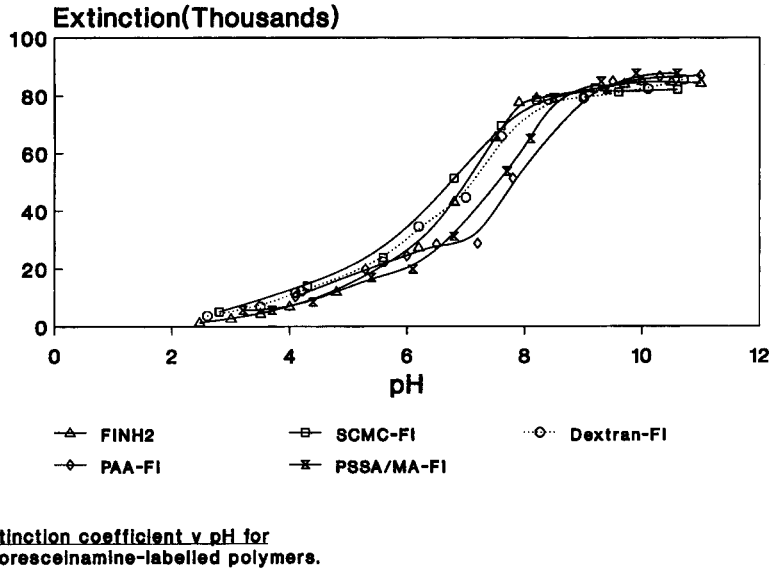


Figure 1 Effective extinction coefficients versus pH for fluorescein amine-labelled polymers: Δ Fla; □ SCMC-Fl; ○ Dextran-Fl; ◇PAA-Fl; X PSS/MA-Fl

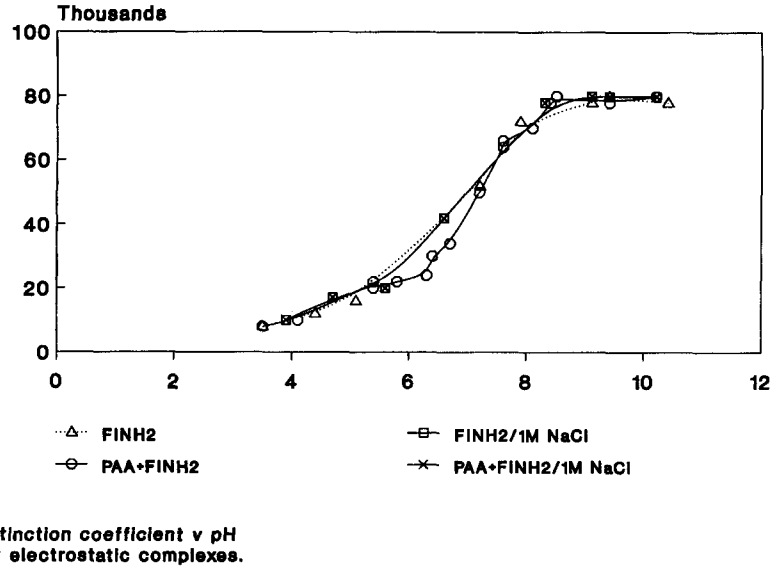


Figure 2 Effective extinction coefficients versus pH for electrostatic complexes: Δ Fla; □ Fla/1 M NaCl; ○ PAA + Fla; X Paa + Fla/1 M NaCl

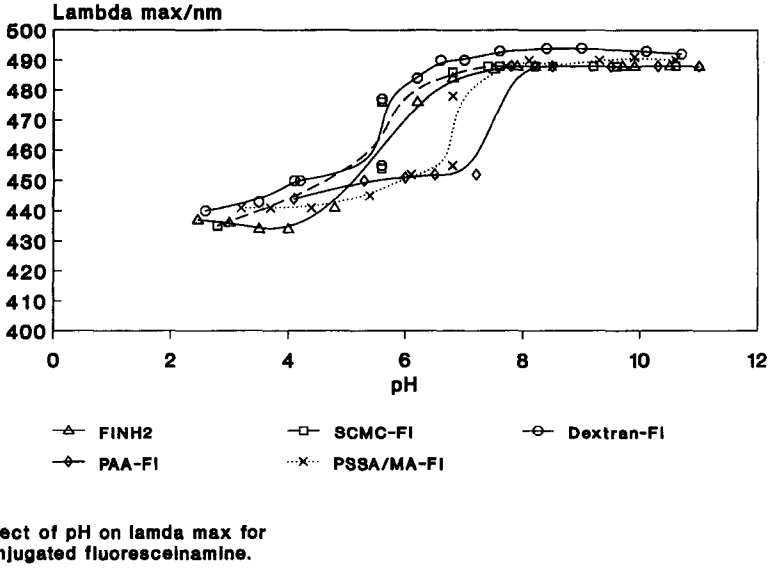


Figure 3 Effect of pH on the position of the band maximum of absorbance for conjugated Fla; Δ Fla; □ SCMC-Fl; ○ Dextran-Fl; ◇ PAA-Fl; X SS/MA-Fl

Table 3 Extinction coefficients for species of fluorescein at various pH

pH	2.2	4.6	6.7	12
Dominant species	1	2 + 3 + 4	5	6
Wavelength of maximum (nm)	435	435	435 + 475	488
Extinction coefficientmol^{-1}cm^{-1}	55 000	16 000	30 000 + 31 000	88 000

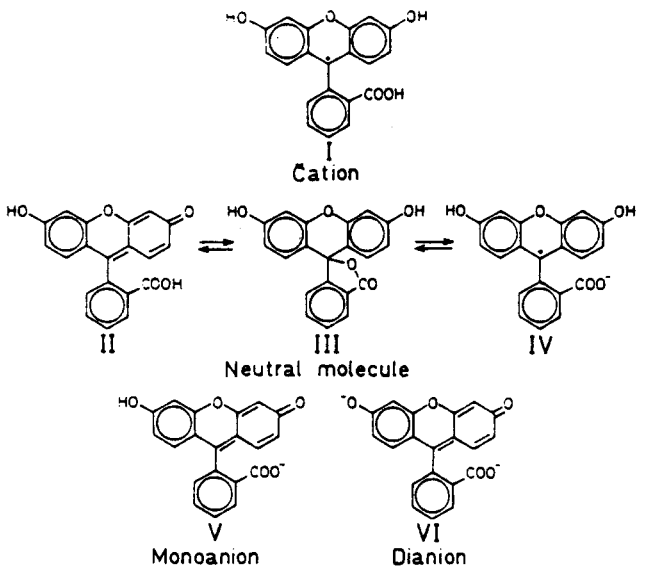
label within the polymer coil. To establish the importance of this, the absorbance of mixtures of unlabelled PAA with Fla were measured and are shown in *Figure 2*. Again absorbances were taken from the maximum in the absorption band, which changed with pH. The extinction coefficient for Fla in the presence of PAA at the intermediate pH (around 7) is lower than in the absence of polymer, though they are the same if 1.0 mol dm^{-3} NaCl is added.

The maximum in the absorption of fluorescein and its derivatives depends on the species present[7] and hence on the pH. The maximum was measured as a function of pH and is shown in *Figure 3*. The species of fluorescein present[8] are shown in *Scheme 1*.

The extinction coefficients at various pH values are shown in *Table 3*, the data being taken from ref. 7.

From *Figure 3* it is apparent that the presence of high charge polyelectrolytes—PAA and PSS—MA-delay the transition of Fla from neutral to anionic species, compared to the presence of neutral to low charge density polymers.

Fluorescence intensities for Fla and several labelled polymers are shown in *Figure 4*. It is clear that attaching the label to SCMC has made no significant difference to the emission intensity of Fla, whereas attachment to dextran and



Scheme 1

PAA has restored the fluorescence in the high pH range, similar to that found for fluorescein.

Whilst Eo-Y has been used extensively for dye-binding and assays for protein[9,10], its use as a fluorescent label is not as common. However, as its fluorescence is less pH sensitive than fluorescein, it can have advantages, even though at high pH it has a lower quantum efficiency than fluorescein. *Figure 5* shows the extinction coefficients of Fla, Eo-Y and EtEo as a function of pH. For Eo-Y and its derivatives, these coefficients are essentially constant above pH 4.2, whereas that of Fla is strongly dependent on pH between 4 and 9. These results for EtEo suggest an alternative labelling route for polymers where pH sensitivity is not needed. As the ethyl group is somewhat distant from the main rings of the molecule, it may be possible to attach PAA to eosin at the site of the ethyl group without significantly altering its fluorescence from that of EtEo. The fluorescence from the Eo-labelled PAA (using ethylene diamine as the link) is shown in *Figure 6* together with that from the labels before attachment to any polymers. The results show that the fluorescence intensities from the eosin labels, either attached to polymer or unattached, are

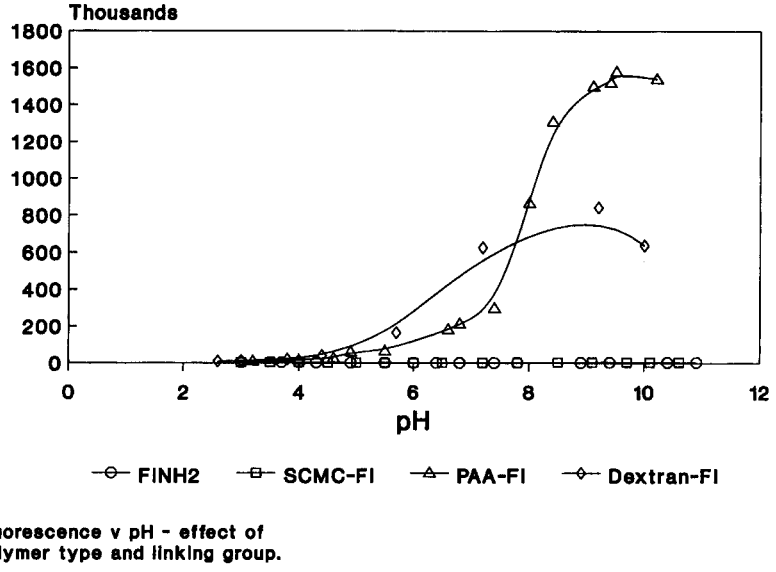


Figure 4 Fluorescence intensity versus pH; effect of polymer type and linking group: ○ Fla; □ SCMC-Fl; Δ PAA-Fl; ◇ Dextran-Fl

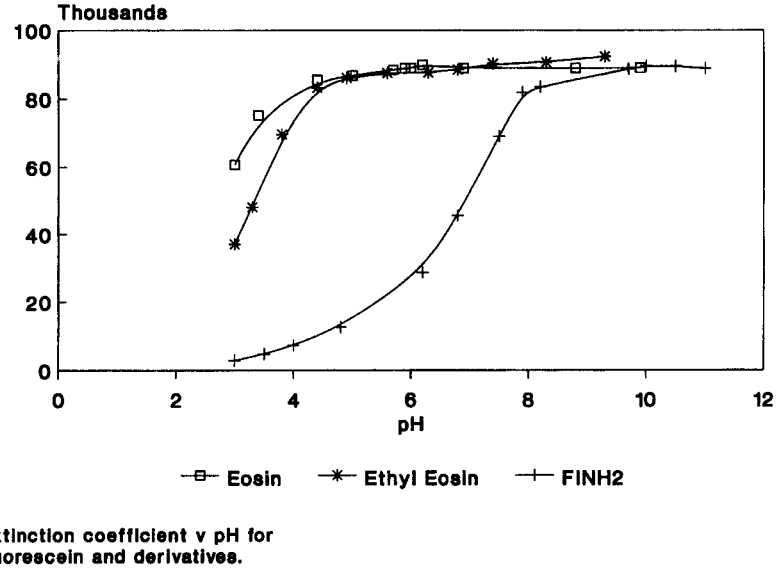


Figure 5 Extinction coefficient versus pH for fluorescein and derivatives: □ Eosin; ☆ Ethyl eosin; + Fla

relatively independent of pH between 4 and 10, whereas the emission of Fl is strongly dependent on pH between 5 and 9. At high pH the quantum efficiency of Fl is about five times that of Eo.

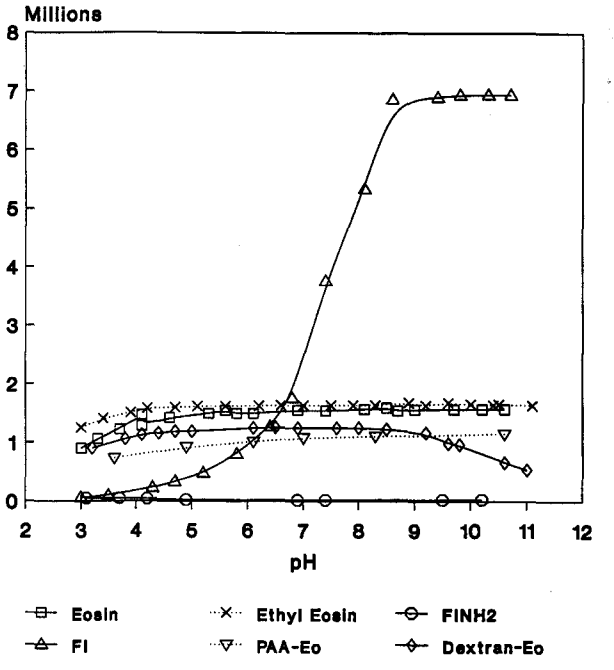
DISCUSSION

The extinction coefficients for the absorption of light by Fla attached to the various polymers can be understood from a consideration of the charge that develops on the label in the presence of the polyelectrolyte. The absorption of light increases when the hydroxyl group of the xanthene ring becomes negatively charged. Ionisable groups have more difficulty in ionising, the closer they are to another charged group, due to the additional electrostatic repulsion this creates. This explains the well known fact that polyelectrolytes, of one charged type, have a pK for ionisation of their groups that increases with extent of ionisation. For the Fla attached to polyelectrolytes, it is evident that as the polymer ionises, it becomes more difficult for the fluorescent group to ionise and move from the neutral species 2–4 to the monoanion and dianion. As species 2–4 have lower extinction coefficients than 5, the increase in the extinction coefficient with increasing pH is delayed in the case of PAA and PSS-MA. Even for the unattached label in the presence of the polyelectrolytes (*Figure 2*), there is a shift in the spectra at pH 6–7. This arises from the Fla being slightly cationic at this pH (the pK_a of the amine[5,7] is about 4.2), thus associating with the anionic polymer and ionising only at higher pH than in the absence of the polymer. When excess electrolyte is added and prevents this association, the extinction coefficient of the unattached label in the presence of the polyelectrolyte returns to the same pH dependence as in the absence of the polymer.

In a similar way, the fluorescence spectra depend on polymer type, though for different reasons to those for the absorption spectra. At alkaline pH the intensity of Fla fluorescence is about 1% that of Fl, due[5] mainly to the back donation of the lone pair of electrons from the nitrogen to the xanthene ring system. Linking groups from this nitrogen to the polymer backbone can interfere with this back donation and thus allow fluorescence to occur to various extents. In *Figure 4* it can be seen that with PAA the fluorescence has been restored, due to the nitrogen in the link being converted from an amine to an amide where the oxygen attracts the lone pair rather than allowing it to quench the fluorescence[5]. With the dextran, the link to the Fla is via the isothiocyanate, which also largely prevents this lone pair back donation to the fluorescein ring and thus gives a fluorescence intensity greater than Fla alone.

For the SCMC where the nitrogen was converted to a secondary amine, the cyanuric chloride attracts[5] very little of the lone pair from the nitrogen, thus giving a low fluorescence intensity similar to that of Fla.

The data in *Figure 5* show that the absorbance of EoY and EtEo is stable over a wider range of pH than that of fluorescein, presumably because of the stabilisation by the four bromine groups. The data show that the groups attached via the phenyl carboxylic acid group appear to have little effect on the fluorescence, probably because of their distance from and lack of conjugation with the ring system. Thus, polymers attached at the phenyl carboxylic group are unlikely to have any through-bond effect on the fluorescence of the EoY label. This is borne out by the data in *Figure 6*, showing that the



Fluorescent emission v pH for fluorescein and derivatives.

Figure 6 Fluorescence emission intensity versus pH for fluorescein and derivatives: □ Eosin; X Ethyl eosin; ○ Fla; Δ Fl; ∇5 PAA-Eo; ◇Dextran-Eo

fluorescence of the EoY-labelled PAA has a similar pH sensitivity and fluorescence yield to that of EoY and EtEo. Similarly, the EoY dextran, labelled via an isocyanate link, shows a fluorescence yield comparable to that of EoY.

The data presented here show that the nature of the link between the fluorescent labels and the macromolecule has a significant effect on both the pH dependence of the absorbance and the fluorescence yield. In addition to this there is a second order effect of charge on the macromolecule retarding the charging of the fluorescein label and hence altering its fluorescence.

CONCLUSIONS

A number of routes to attaching fluorescein and eosin labels to water soluble macromolecules has been studied, together with their effects on absorbance and fluorescent spectra. Fluorescein amine is often used as the label, since the amine group provides a useful reactive site. However, no fluorescence is observed if the linking mechanism does not prevent quenching by the lone pair of electrons. This quenching can be prevented by using an amide (via Woodward's reagent) or isothiocyanate as the linking group between the label and the macromolecule. This also largely preserves the well known pH dependence of the fluorescence of fluorescein. Eosin provides a relatively constant fluorescence over a wider pH range than fluorescein and can be linked to macromolecules via the carboxyl group without significantly influencing this pH stability.

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