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Near-i.r. fluorescent labels for determination of functional groups in polymeric materials

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Cyanine-based near-i.r. fluorescent probes have been applied as labels to determine functional groups in polymeric materials. The advantage of such probes is that they can be applied to polymers which are autofluorescent or strongly coloured or to strongly scattering polymer emulsions. Two examples are discussed: the labelling of a polymer latex with aldehyde functional groups, by application of a cyanine with a hydrazide functionality, and the labelling of amino functional groups in a polyamide utilizing a cyanine label with an isothiocyanate functionality. The cyanines which have been applied absorb and fluoresce in the 650–700 nm range and in the 750–800 nm range, respectively. In addition to near-i.r. fluorescence, the cyanine dyes have strong absorption (with extinction coefficients in the order of 2×10^5 l mol^{-1} cm^{-1}). It is shown that by application of reflection absorption measurements also sensitive detection of labelled functional groups can be realized. The combined application of fluorescence and reflection measurements allows for the detection of concentration and distribution of functional groups in polymeric systems down to very low levels. © 1997 Elsevier Science Ltd.

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INTRODUCTION

Determination of distribution and concentration of functional groups in polymers and on polymer particles is a very important aspect of polymer analysis. Properties such as stability, entanglement, solubility, possibilities for further reactions and processability are strongly influenced by the presence and nature of functional groups. Functional groups on surfaces are a major determinant of properties of materials used in a variety of applications. A few examples are: (1) particles used in chromatographic systems, where functional groups may provide specific interactions with particular analytes, (2) latices used for diagnostic purposes, where particles are bound to antibodies to invoke immunochemical diagnosis, (3) functionalized latices applied in coatings with improved properties, such as better blocking or water resistance and (4) modified fibre surfaces with special adhesion properties. For the study of the relationship between the structure of the surfaces of these particles and their activity, important information can be derived from the concentration and distribution of the functional groups.

A number of analytical techniques is available to determine the concentration of functional groups. Most of these have been developed for homogeneous systems; in some cases they can be applied to heterogeneous systems as well, provided that the concentration of the functional groups is sufficiently high. Direct measurements are based on titrations, using a variety of detection principles (potentiometry, conductivity, colorimetry, etc.)[1]. Alternatively, strongly coloured (absorption) or fluorescent probes can be used, which form specific, covalent, bonds with the functional groups. The application of such probes, which allow for spectroscopic determination of the functional groups, is well known from biomedical as well as chromatographic applications, but only recently has been used to study polymeric materials[2]. In homogeneous systems the application of covalent labelling is straightforward, provided that the unreacted label does not interfere: removal of unreacted label may require extensive chromatographic sample pretreatment[3–6]. In heterogeneous labelling, however, the use of absorption and fluorescence labels is not straightforward. Due to the presence of strongly scattering particles, simple transmission or fluorescence measurements are not possible. Special detection techniques, which minimize the influence of light scatter, have to be

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developed. In addition, many polymers are coloured or show autofluorescence, which may complicate the application of such 'spectroscopic' labels. On the other hand, the removal of excess label in heterogeneous systems in general is simpler than in homogeneous solutions.

In this paper the application of labels, especially developed to circumvent the complications related to scattering and autofluorescence outlined above, will be discussed. The specific aspect of the labels is that they absorb and fluoresce in the near-i.r., in particular beyond 650 nm. Such near-i.r. fluorescent labels have several advantages over commonly used labels[7,8]. Firstly, their electronic transitions are located in a spectral region where most polymeric systems are optically inert, i.e. interference by autoluminescence or absorption of polymers or by inhomogeneities in these polymers is negligible. Many polymers (for instance polyamides, polyaramides and polyethers) show absorption in the u.v. and blue and luminescence in the blue and green part of the spectrum, respectively. Secondly, since for small particles the scattering efficiency varies with the fourth power of the illuminating wavelength, much sensitivity can be gained by moving the excitation wavelength from 300 to 650 nm, or even further, to reduce the background signal.

The labels considered in this paper are cyanine dyes; they have only a slight change in configuration between ground state and excited state, which on one hand leads to a small Stokes' shift, but at the other to an extremely high absorption coefficient (in the order of 200.000 l mol^{-1} cm^{-1}, at least 10-times higher than that of most polyaromatic organic molecules)[9]. In particular the high absorption coefficients make the near-i.r. dyes also good candidates for applications in labelling and imaging, and for use in absorption/reflection assays.

A complicating factor in the use of fluorescent probes in polymeric systems is that most labels have been developed for biomedical applications. This implies that most labels synthesized until now give good covalent bonds in solvents like water, but that their reactivity in good solvents for polymers is far from optimal. For this reason, first some exploratory experiments have to be performed to determine the optimum reaction conditions of the labels and the functional groups in the polymers in the latter kind of solvents.

EXPERIMENTAL

Preparation of labels; labelling reactions

Latex aldehyde labelling. Latices provided with different concentrations of aldehyde-groups were prepared by oxidation of vicinal-OH containing emulsions with specific amounts of periodate. The latices are prepared by core–shell emulsion polymerization using polystyrene as core and glycidylmethacrylate as shell[10]. Vicinal-OH groups are prepared from the epoxide-functionality of glycidylmethacrylate by acid hydrolysis. The concentration of aldehyde groups can be determined by titration with hydroxylamine–hydrochloride; the liberated hydrochloride is titrated with base. This technique is only applicable for rather high concentrations of functional groups. For lower concentrations the periodate concentration is varied and the concentration of aldehyde-groups is obtained by extrapolation. In the present study the cyanine dye depicted in *Figure 1a* has been used. This label is excited at 600–700 nm and emits at around 720 nm. The preparation of the label, which contains a hydrazide functionality is described in ref. 11. Labelling is done by stirring the latex for 1 h at 50°C (pH 3) with an at least two-fold excess of the hydrazide label. When the labelling procedure has been completed, excess label is removed by extensive microfiltration with methanol and, subsequently, with water. It is vital that no epoxide-groups remain after preparation of the vicinal-OH groups, since also epoxides may react with the hydrazide label. Careful hydrolysis in methanol ensures quantitative conversion of the epoxides into vicinal-OH groups, also for the epoxide groups which are not easily accessible in aqueous dispersion.

Polyamide labelling. Polyamides prepared from caprolactam (i.e. nylon-6) are only sparingly soluble in a limited number of solvents: acids like formic acid or hexafluoroisopropanol (HFI), or the solvents *N*-methylpyrrolidone (NMP; in the presence of lithium chloride, to break hydrogen bonds in the solid polymer) and *m*-cresol. They contain primary amino-groups and carboxylic acid groups as end groups. To determine the reaction rates of isothiocyanate fluorophores in these solvents, first model reactions were performed for labelling of primary amino-(end)groups in polyamides using butylamine as substrate. Next, the reactions of polyamides with phenylisothiocyanate in phenol-d_6 were followed by n.m.r. at 60°C. Subsequently, a reaction was performed according to the optimized conditions, during 8 h at 60°C using the cyanine label shown in *Figure 1b*. The label was prepared as reported in ref. 13. The dye, which contains a seven-membered conjugated polyene-bridge, i.e. one C=C unit longer than the label applied for the labelling of the aldehyde groups, is excited in the 700–780 nm range, and it emits at around 800 nm. After homogeneous labelling the excess label can be easily removed by a washing procedure, during which the labelled polymer is procured as precipitate.

Some experiments were done with the visible dye Lucifer Yellow, which can be utilized for labelling of both amino-groups (VS, vinylsulfonate form) and of aldehyde-groups (CH, hydrazide form).

Spectroscopic measurements

^{1}H and ^{13}C n.m.r. measurements of solutions containing primary amino-groups, both as monomer and as polymer solution, were done at elevated temperatures, using a Bruker AC300 300 MHz n.m.r. spectrometer.

Diffuse reflection measurements were performed on particles labelled with Lucifer Yellow and after reaction with the hydrazide functionalized cyanine dye, using a Varian Cary 5 absorption spectrophotometer equipped with a Praying Mantis accessory. This accessory is designed to obtain reflection data using normal incidence irradiation and detection of the diffusely reflected excitation light over a rather wide scattering angle collected by a pair of large parabolic mirrors. The reflection measurements were done on an 'infinitely' thick layer, so that the absorption could be approximated via the Kubelka–Munk relation[12]. For the very concentrated samples the material was diluted with blank material to remain in the linear concentration range for the Kubelka–Munk application.

Fluorescence emission and excitation spectra were recorded for polymers labelled with both cyanine dyes. The spectra were recorded in a PTI Alphascan fluorescence spectrophotometer on diluted emulsions. Emission

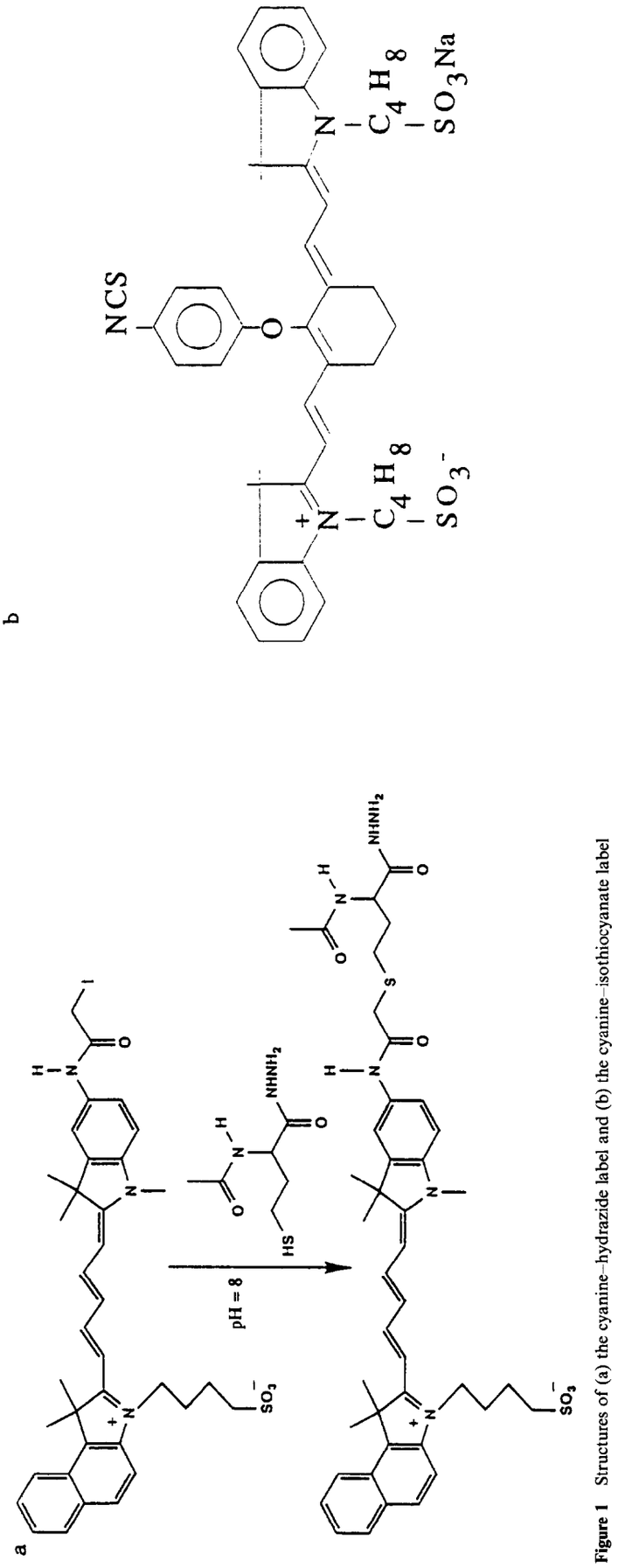

Figure 1 Structures of (a) the cyanine–hydrazide label and (b) the cyanine–isothiocyanate label

was detected in a front face configuration, under an angle of 20° of the excitation light. For the isocyanate label the excitation spectra were measured by monitoring the emission at 835 nm; the emission spectra were obtained by excitation at around 700 nm. For the hydrazide label, with the shorter conjugated polyene-chain, emission was monitored at 750 nm, excitation was done at 640 or 700 nm. Lucifer Yellow excitation spectra are monitored at 535 nm, emission spectra were recorded using excitation at 435 nm. Bandpass was 1.5 nm for excitation and emission. In the near-i.r. experiments either a Hamamatsu R928 or a near-i.r. sensitive Hamamatsu R2658 photomultiplier were used, thermoelectrically cooled to −20°C.

Materials

Lucifer Yellow VS and Lucifer Yellow CH were obtained from Aldrich. Both cyanine labels were synthesized as described in the literature[11,13]. Polyamide samples and core–shell latices have been prepared according to documented procedures[14]. All solvents used were spectrophotometric grade.

RESULTS AND DISCUSSION

Labelling of latices

Latices with aldehyde functionalities in the surface layer are applied for covalent bonding of proteins and antibodies for diagnostic tests. To be able to evaluate the concentration and distribution of aldehyde groups in the core–shell latex several techniques can be used. In particular, by titration in different solvent systems the concentration both in the surface layer and across the latex can be determined. The titration method, however, has its limitations, in particular when one is interested in the low concentration range and in the fraction of functional groups which is accessible for larger molecules, i.e. the relevant functional groups for most applications. Optical techniques are very suited for this purpose[2]: reflection absorption methods and fluorescence methods can be straightforwardly applied. However, labelling with dyes which absorb and fluoresce in the u.v. and visible range is not appropriate due to significant effects of scatter by the latex particles. The use of near-i.r. dyes may provide a good alternative. The cyanine dyes, which are well documented in the literature[7], have high extinction coefficients and have relatively high fluorescence quantum yields and hence are very suitable for labelling of functional groups on particles.

For labelling of aldehyde groups a cyanine dye was synthesized containing a hydrazide functionality (see *Figure 1*). The label could be synthesized with good yield from the iodoacetamido precursor, prepared according to Mank *et al.*[5], by reaction with a suitable thiolhydrazide. Other, more direct, routes of synthesis appeared unsuccessful, mostly due to the instability of the cyanine moiety under extreme reaction conditions[11]. Initial tests of the reactivity of hydrazine label were done on a mixture of aliphatic aldehydes in homogeneous solution. The labelling appeared to be quantitative under the applied reaction conditions (within 10 min at room temperature in 7/3 acetonitrile/acetic acid). The labelled aldehydes could be separated easily on a C_{18}-chromatographic column with an acidified (0.3% trichloroacetic acid) 1/1, v/v, acetonitrile/water eluent. In line with results reported by Mank *et al.* the separation efficiency

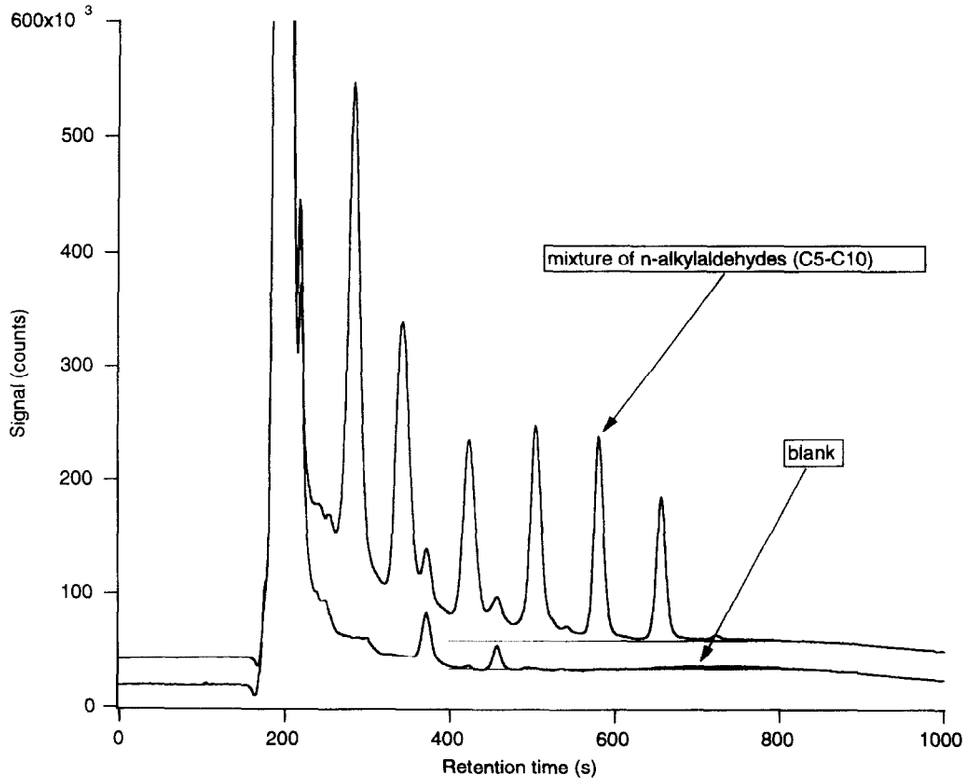

Figure 2 Chromatogram of n-alkylaldehydes (C_5–C_{10}) labelled with the cyanine–hydrazide. Detection with an absorption detector set at 665 nm

is good[5], which is surprising in view of the large size of the label as compared to that of the analyte, indicating the good solubility of the label in the polar mobile phase. The chromatogram obtained by detection with an absorption detector at 665 nm is shown in *Figure 2*. The large peak at zero retention is due to unreacted label, which is always difficult to remove quantitatively in homogeneous labelling applications.

The results obtained in the homogeneous phase give confidence as to the possibilities to label the water dispersed particles with aldehyde functionalities in the heterogeneous system. Latices with a solid content of 1% were readily labelled at 50°C during 1 h at pH 3. Quantitative reaction of the aldehyde groups which are accessible to the label is obtained for an at least two-fold excess of the cyanine dye. Probably the local concentration of the label is much larger, though, as is suggested by comparison to the results obtained for the Lucifer Yellow CH label. The Lucifer Yellow has much better water solubility than the cyanine label. For a reaction of both labels with the latex, the percentage of labelled aldehyde groups (as compared to the titrated total amount of aldehydes) is almost an order of magnitude lower when the highly polar Lucifer Yellow label is used, than in the reaction with the cyanine dye. In the latter labelling it is estimated that about 20% of the total concentration of aldehyde groups is reached by the reactive dye and is labelled. Just the accessible aldehyde groups are relevant for the diagnostic application of the latices, where the functional groups act as anchor for antibodies or antigens. The titration method thus significantly overestimates the concentration of functional groups in the particles. Reflection absorption spectra and fluorescence spectra of cyanine labelled latices with different concentrations of aldehyde groups are shown in *Figure 3*. The reflection spectra indicate that the cyanine groups on the latices form dimers or aggregates for particles with high ($>1\ \mu eq\ m^{-2}$) concentrations of aldehyde functionalities[15]. In the spectra the dimers show blue shifted absorptions. The lower concentrations of aldehyde-groups yield spectra with the same appearance as dissolved cyanine monomers. The Kubelka–Munk reflection spectra shown in *Figure 3a* have absorptions which vary linearly with the concentration of aldehyde-groups in the region where the monomer absorptions are visible. In this region, which ranges from 0 to $0.2\ \mu eq\ m^{-2}$, fluorescence techniques can also be successfully applied (see *Figure 3b*).

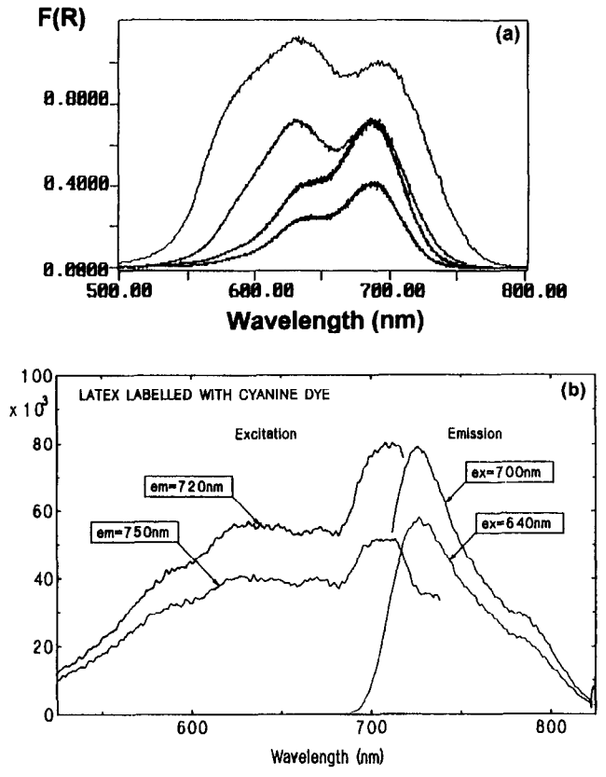

Figure 3 (a) Kubelka–Munk reflection curves of aldehyde-functionalized core–shell latices labelled with the cyanine–hydrazide label; from top to bottom $7\ \mu eq\ m^{-2}$ (diluted 1 : 9 with blank latex), $1.5\ \mu eq\ m^{-2}$ (diluted 1 : 4 with blank latex), $0.44\ \mu eq\ m^{-2}$ and $0.22\ \mu eq\ m^{-2}$. (b) Fluorescence emission and excitation spectra of aldehyde functionalized latex labelled with cyanine–hydrazide label. The aldehyde concentration was $0.44\ \mu eq\ m^{-2}$

The dimers have low fluorescence quantum yields and are not observed in the emission spectrum. The Stokes' shift of the fluorescence spectra is very small, which is typical for cyanine dyes. A small Stokes' shift implies that the geometry of the ground and excited states of the cyanine compound is very similar. In general such conditions lead to very strong 0–0 transitions in emission and high extinction coefficients in the absorption spectrum. Further detailed analysis of spectra and labelling conditions will provide data on quantitative analysis with the cyanine–hydrazide label and will result in estimates of the distribution of label in the surface layer of the latex particles.

It appears that excess label can be removed from the latex, by application of microfiltration techniques. The efficiency of the extensive washing procedure has been checked by examining the dye content of the rinsing liquid by fluorescence measurements. Removal of the unreacted dye is realized by subsequent washing with methanol and water. The filtration procedure is sustained until no dye can be detected in the washing liquid, which in general is realized overnight. The ease of removal of excess dye is an important advantage of the application of fluorescent labels in heterogeneous systems. To make sure that only the aldehyde functionality is quantified it is vital to remove any remaining epoxide groups from the particles, for carrying out the reaction for longer periods of time leads to reaction of the label with the epoxide groups. The epoxides are effectively removed by acid hydrolysis.

Labelling of polyamides

The labelling of latex described above can be performed according to conditions as described in the literature for hydrazides applied in biochemical reactions, i.e. in aqueous solutions. Unfortunately, water is not a suitable solvent for labelling of polyamides. Solvents which are appropriate for dissolution of polyamides are more exotic, like formic acid, HFI, NMP or *m*-cresol. To make sure that the amino endgroups are quantitatively labelled the accessibility of these groups is essential. Therefore, the labelling reaction preferentially should be carried out in the homogeneous phase. Since no information on reactions of isothiocyanates in the solvents mentioned above was available from the literature, first model reactions were performed for reaction of phenylisothiocyanate with

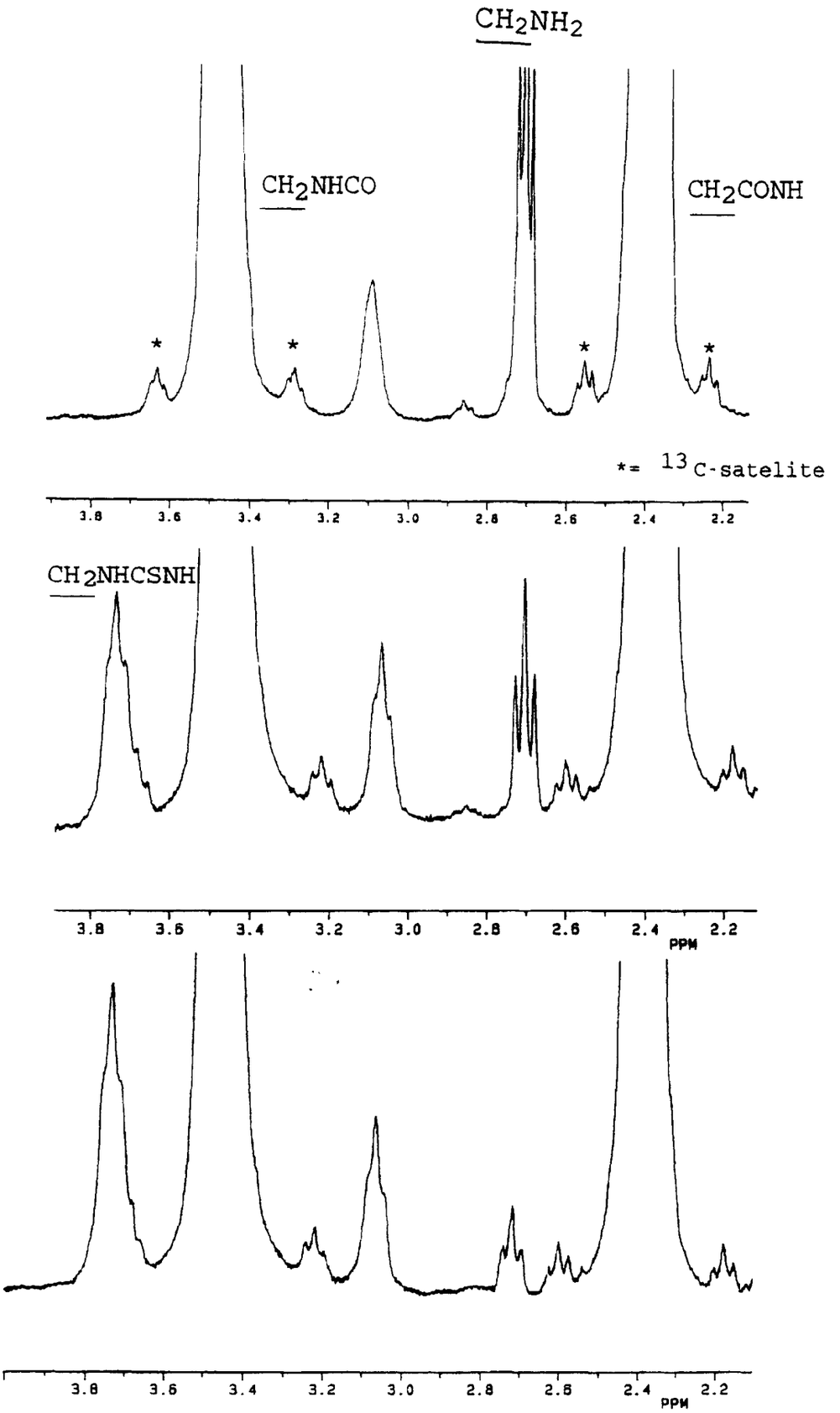

Figure 4 ^{13}C n.m.r. spectra of the reaction products formed during the interaction of phenylisothiocyanate and polyamide. From top to bottom the spectra obtained after 0, 13 and 26 min of reaction at 60°C are shown. After 1 h more than 90% of the amino-groups has reacted

butylamine and later with a polyamide. It appeared that no reaction was observed in both acidic solvents, formic acid and HFI, probably because of the protonation of the amino-group. In NMP or DMSO the reaction proceeds, but a disadvantage of NMP as solvent is the necessity to add an appreciable amount of LiCl to break the hydrogen bonds between the polyamides in the solid polymer, which complicates their dissolution. The high salt content makes the clean-up of the reacted polymer more difficult. In *m*-cresol (n.m.r. experiments in phenol-d_6 at elevated temperature) the reaction proceeds rapidly and quantitatively, as is shown in *Figure 4*. It was therefore decided to perform the labelling reactions in this solvent.

The cyanine–isothiocyanate label reacts readily with the dissolved polyamide: 4 h heating at 50°C is sufficient to obtain a quantitative reaction. Following the reaction, removal of excess label was done via a washing procedure. The reaction product gives the fluorescence emission and excitation spectra shown in *Figure 5*. The product gives a negligible Stokes shift, as expected for a

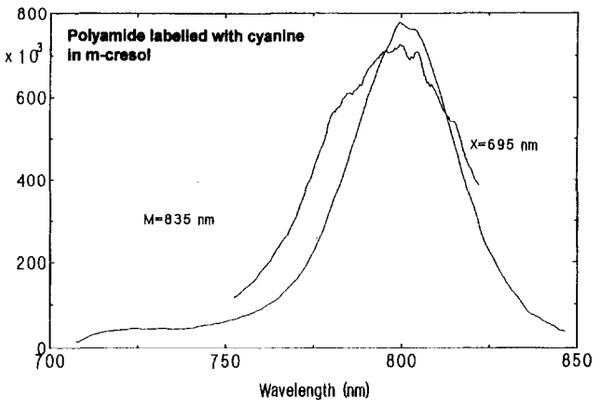

Figure 5 Fluorescence emission (excited at 695 nm) and excitation (monitored at 835 nm) spectra of polyamide labelled with the cyanine–isothiocyanate label. Spectra were recorded in *m*-cresol

cyanine dye. Again the extinction coefficient of the cyanine dye is very high, the fluorescence quantum yield of the cyanine label applied here is much lower than that of the cyanine label applied for the aldehyde labelling, though (less than 0.1 as compared to about 0.25 for the latter label). The cyanine–isothiocyanate label has a polyene chain which is one double bond longer than the one applied for the aldehyde labelling. As a consequence the 0–0 transition is shifted about 100 nm to the red, which is favourable since the background is even further reduced, but is unfavourable for the fluorescence quantum yield: radiationless transitions will be enhanced in a compound with a smaller energy gap between excited state and ground state. Also, the longer conjugated polyene chain in the cyanine–isothiocyanate label is intrinsically weaker than the shorter chain in the cyanine–hydrazide label. To stabilize the longer polyene chain the central ethylene-bond is protected by incorporating it in a six-membered ring.

Upon addition of acid (in the form of formic acid, HCOOH) the absorption spectra of the label bound to the polyamide change significantly: *Figure 6* shows absorption spectra of the labelled solution with different amounts of acid added. The absorption at 790 nm disappears, whereas a new absorption at about 600 nm is observed. In HFI similar effects are observed. The acid solution also shows fluorescence, blue shifted just as the absorption spectrum. Two fluorescent species are present in the acid solution, as can be inferred for the emission spectra obtained for different excitation wavelengths, depicted in *Figure 7*. One emission spectrum, with its main peak at 555 nm, is obtained by excitation at 480 nm. The other emission spectrum peaks at 630 nm, and is effectively excited at 550 nm. The absorption spectrum in the orange part of the spectrum shows several maxima as well. Addition of base (sodium hydroxide) did not result in restoration of the previously obtained spectra. The most likely explanation for the spectral changes is an attack on one of the double bonds in the central polyene chain, leading to chain scission and the formation of two fluorescent moieties.

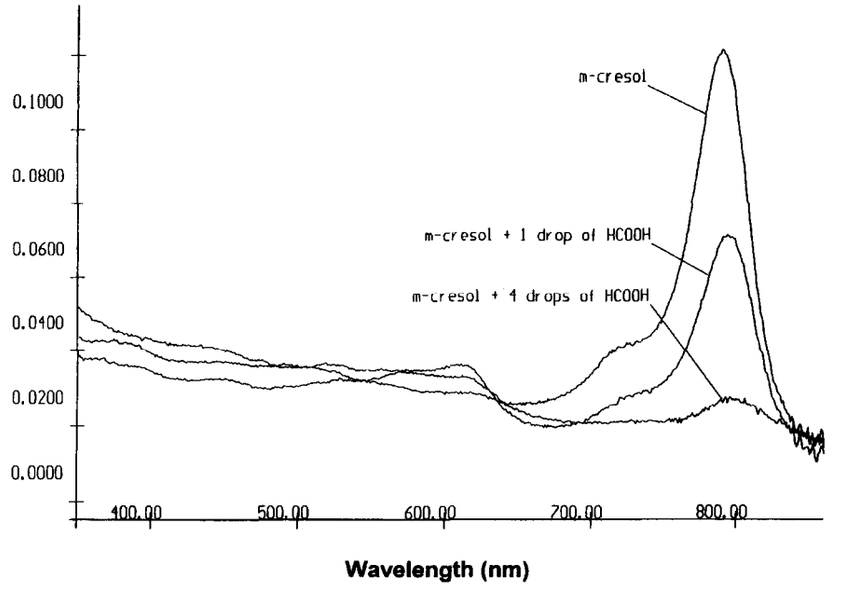

Figure 6 Absorption spectrum of polyamide labelled with the cyanine–isothiocyanate label in *m*-cresol, acidified with formic acid

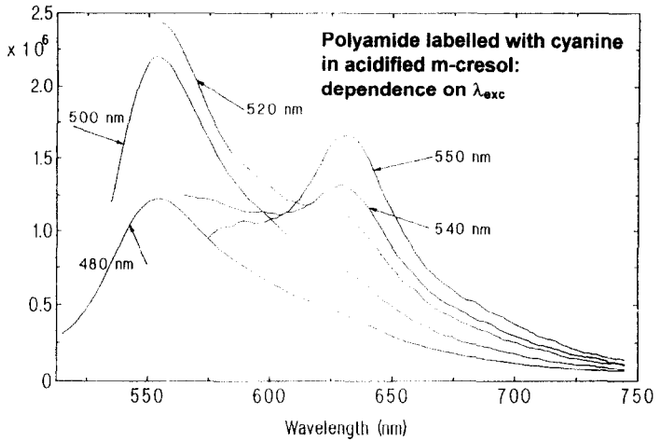

Figure 7 Fluorescence emission spectra of polyamide labelled with the cyanine-isothiocyanate label in acidified *m*-cresol, obtained by application of different excitation wavelengths

The unreacted label, also in *m*-cresol, was submitted to the same treatment as the labelled polyamide. However, even when significant amounts of formic acid were added to the *m*-cresol solution no reduction of the 790 nm absorption was observed, apart from a small effect attributable to dilution of the original solution. The difference of the effect of acidification on the free and the reacted label is striking. The observations for the labelled nylon supports the assumption that the nylon indeed has reacted with the isocyanate label. Apparently, the chain scission does not take place in the dissolved, unreacted, label. Further work is necessary to fully elucidate the observations, but on the basis of the present information it is recommended to be very careful when applying the cyanine dyes in acidic solutions. The observations argue—again—not to perform the labelling reaction in one of the acidic solvents, but also not to use an acidic solvent to dilute or dissolve the labelled products.

CONCLUSIONS

Near-i.r. fluorescent labels have been prepared which react with polymeric particles and polymers in solution. The fluorescence spectra in the near-i.r. are not disturbed by background signals or scatter effects. The exploratory experiments described in this paper indicate that functional groups in both systems investigated may be determined down to very low concentrations, so that relatively small amounts of samples with medium to high concentrations of endgroups can be studied, or, alternatively, trace amounts of endgroups (e.g. unreacted endgroups in a protein loading reaction) can be determined in normal sample quantities.

Since the near-i.r. labels have high extinction coefficients, also reflection absorption measurements can be used to determine endgroup concentrations. These measurements in principle extend the region in which quantitative determinations can be done to about 1 μeq m^{-2} for the latices. At higher concentrations additional, blue shifted, absorption peaks are observed in the spectra, which are probably due to dimers and/or aggregates of the attached groups. On the particle surface the side groups are very close together, so that they may easily interact. From the concentration dependency of the (change in) reflection spectrum the interactions can be studied; they may provide information on the relative distances of the chromophores. In the polyamide solution no aggregation effects are observed. This can be attributed to the fact that in the chosen solvent the polyamide is well dissolved, so that all side groups are surrounded by solvent molecules. If the interaction between the fluorophores and the solvent molecules is strong, the formation of dimers will be more difficult.

Since most reports in the literature focus on the application of cyanine- (and other) labels in aqueous solutions, of interest for mainly biomedical applications, it is necessary to study and optimize the reactivity of such dyes in the more extreme solvents which are needed for labelling of polymers. This can be done most easily by making use of n.m.r. spectroscopy. When cyanine dyes are utilized one must be careful with the application of acidic solutions, since decomposition of the isocyanate label after reaction with polyamide has been observed.

An important advantage of labelling techniques applied to heterogeneous systems, such as particles or surfaces in general, over the mostly applied labelling in homogeneous systems, is that unreacted label can be removed effectively by simple (micro)filtration approaches. In homogeneous systems removal of excess label in general requires careful chromatographic separation, extraction procedures or dissolution/precipitation, with the risk of loss of material. Such complications can be circumvented in heterogeneous systems; however, one has to be aware of possible spurious labelling due to aspecific interactions.

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