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A new reactive polymer suitable for covalent immobilisation and monitoring of primary amines

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Abstract

A new polymer capable of reacting with primary amines was synthesised from allyl mercaptan, *o*-phthalic dialdehyde and ethylene glycol dimethacrylate by radical polymerisation. Reactive hemithioacetal formed by allyl mercaptan and dialdehyde can bind primary amino groups without additional pre-activation forming the fluorescent isoindole complex. It gives a great opportunity to monitor binding and perform loading of the amino compounds onto the reactive surface. The reactive polymer is found to be an effective matrix for immobilisation of the proteins and other amino-containing compounds in affinity chromatography and could be used for their detection in solution. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Recently the development of the so-called 'smart' polymers has attracted great attention in the field of biotechnology due to their ability to 'sense' the change in the environment or to change predictably the environment characteristics. 'Smart' polymers are used in bio-separation and drug delivery, for the development of new biocatalysts, as biomimetic actuators and as surfaces with switchable hydrophobic–hydrophilic properties [1]. There is a strong interest to utilise 'smart' polymers in the field of ecology where environmentally sensitive polymers can control or even prevent the bacterial contamination of the solid surfaces in a non-sterile environment [2]. Food industry also creates a potentially big market for 'smart' polymers where they can be used for selective removal of undesirable components, separation and analyses [3].

The paper presented here describes the synthesis and analysis of new 'smart' polymers which are able to react with primary amines such as ammonia and amino acids, peptides, proteins and nucleic acids. The polymer reactivity is based on a well-known reaction between primary amines, *o*-phthalic dialdehyde (OPA) and β-mercaptoethanol in solution [4]. Homologous aromatic dialdehydes such as OPA, P-2331, naphthalene-2,3-dicarboxaldehyde (NDA, N-1138) and anthracene-2,3-dicarboxaldehyde (ADA, A-1139) are essentially non-fluorescent until reacted with a primary amine in the presence of excess of mercaptan, such as 2-mercaptoethanol, to yield a fluorescent isoindole (Fig. 1). The increase in fluorescence emission permitted monitoring of the binding events and quantification of the concentration of primary amino compounds in solution [5]. Heterogeneous assay based on amino-containing polymer and OPA (P-2331) was used to monitor the concentration of mercaptoethanol and sialic acid in solution [6]. Despite the high sensitivity achieved in those studies, the necessity to use a multicomponent mixture for analysis limits the application of this method. Recently the synthesis of a new polymer, which already contains two of the three components: dialdehyde and mercapto group, necessary for fluorescence complex formation was described [7]. An organic soluble reactive polymer (RP) containing hemithioacetal was prepared as a self-assembled layer on the transducer surface and used as a reactive 'reagent-free' coating for the immobilisation of the amino group-containing compound. A wide variety of monomers, which can be used for tuning of the polymer properties, could bring different practical applications. Here we describe the synthesis and investigation of highly cross-linked polymers containing hemithioacetal. These materials can be used in affinity chromatography for protein and DNA immobilisation and in sensor technology for detection of primary amines.

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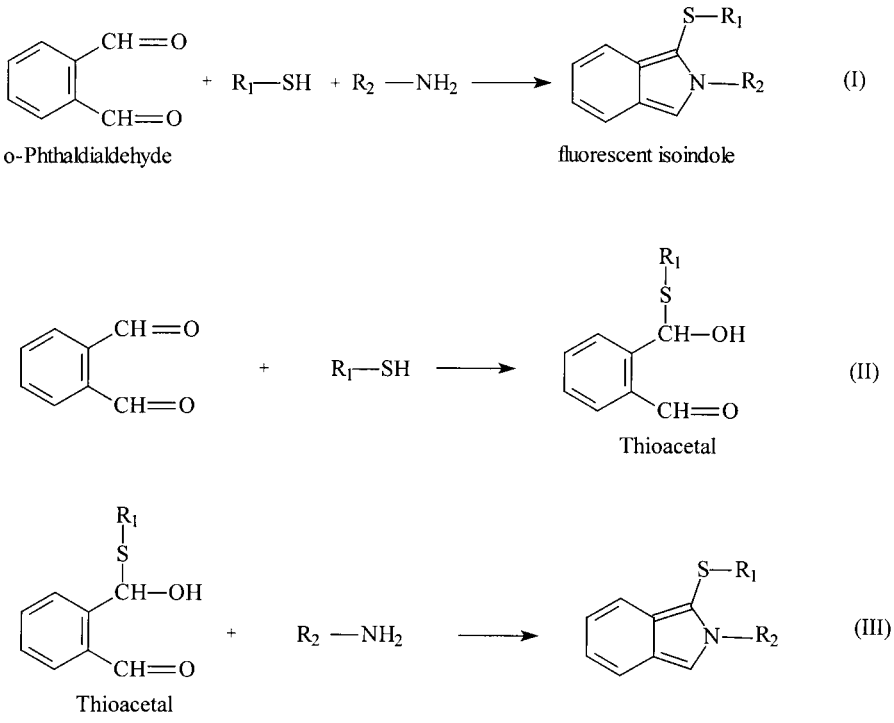

Fig. 1. Interaction between dialdehyde, mercaptan and primary amine (I), hemithioacetal formation (II), formation of the fluorescent isoindole complex between hemithioacetal and primary amine (III). R_1–SH — polymerisable mercaptan (AM), R_2–NH_2 — primary amine.

2. Experimental

All compounds were obtained from commercial sources and were analytical or HPLC grade. Ethylene glycol dimethacrylate, acetonitrile, allyl mercaptan (AM), asobis (isobutyronitrile) (AIBN), horseradish peroxidase (HRP), bovine serum albumin (BSA), haemoglobin were purchased from Sigma, OPA, cytochrome C from Aldrich, bicinhonic acid (BCA micro reaction kit) from Pierce and microperoxidase was bought from Biozyme Laboratories (UK).

2.1. Preparation of the reactive polymer

Polymer was prepared by mixing together 10 mmol (1.98 g) of ethylene glycol dimethacrylate, 1 mmol (134 mg) of OPA and 2 mmol (148 mg) of AM and acetonitrile (2 ml). Monomer mixture was thoroughly purged with nitrogen. Polymerisation was initiated by adding 50 mg of AIBN and heating overnight at +80°. Polymer was ground and washed in acetone. Polymer particles of size 1–5 μm were collected by seiving and decantation.

2.2. Fluorimetry with reactive polymer

The experiment was performed as follows: 9 mg of synthesised polymer were suspended in 3 ml of 0.1 M sodium phosphate buffer, pH 8.0 and its excitation and emission was measured in 3 cm^3 quartz cuvette using RF-5301 PC spectrofluorophotometer (Shimadzu, Japan). To investigate the formation of isoindole, aliquots of concentrated NH_4OH solution was added to the polymer suspension. A change in fluorescence was recorded as a function of time (excitation wavelength E_{ex} = 355 nm). All measurements were made in triplicate.

2.3. Protein immobilisation

The binding capacity of the polymer was demonstrated with several proteins: microperoxidase (FW = 1 kDa), cytochrome C (FW = 12.4 kDa), HRP (FW = 44 kDa), BSA (FW = 66 kDa) and haemoglobin (FW = 67 kDa). 10 mg of polymer was incubated with 400 μl of protein solution (5 mg/ml) in 10 mM HEPES buffer, pH 8.6 for 18 h. The protein concentration before and after sorption was measured spectrophotometrically using the BCA method [8] and calculated using calibration curves obtained individually for each protein.

3. Results and discussion

The hemithioacetal-containing polymer was synthesised as described previously [6]. Accordingly to elemental analysis 42% of the AM was included in the synthesised polymer. Incomplete polymerisation can be explained by low reactivity of allyl groups in general as well as inhibiting properties of mercapto group in radical polymerisation. Although we used an excess of AM in comparison with OPA to make the polymer, IR spectrum of the resulting material had much weaker S–H stretch than anticipated. It is possible to

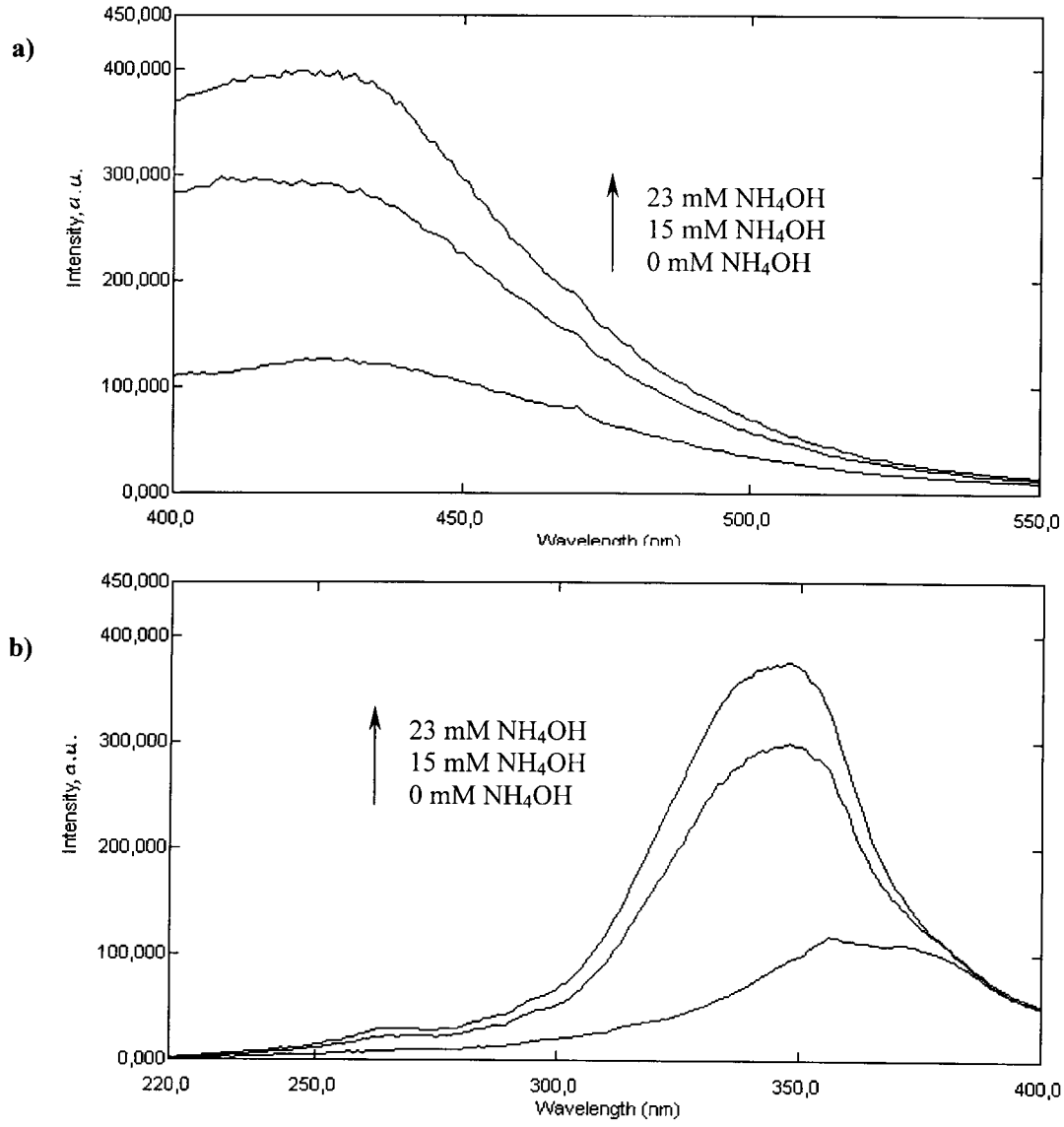

Fig. 2. Changes in the (a) emission and (b) excitation spectra of the polymer in response to the addition of NH_4OH.

conclude that the majority of sulphur in the polymer exists in the form of hemithioacetal or thioether.

The ability of the polymer to react with primary amines was studied measuring the change in fluorescence upon addition of spiked concentration of ammonium hydroxide solution. Fluorescent measurements were carried out with a polymer suspension with particle size of 1–5 μm, which has good stability in water. It was found upon addition of the 23 mM of NH_4OH the intensity of the fluorescent excitation and emission of the polymer suspension was three times higher (Fig. 2). The excitation and emission maximum were 347 and 430 nm, respectively. Fig. 3 shows the kinetics of the isoindole formation in polymer suspension. For practical purposes when faster assay is desirable the signal can be measured in 30 min when its magnitude reaches 70% of the maximum. This kinetics is similar to the well-established reaction of isoindole formation in solution applied extensively for the detection of many primary amino analytes [5].

Different pH and buffer concentrations were used to find the optimal conditions for the reaction between polymers and primary amines. In the reaction with NH_4OH it was found that the optimum pH is 8.0 (Fig. 4). The influence of buffer concentration was less pronounced. Practically no difference was found for buffers with 10 and 200 mM concentrations (data not shown).

Progressive increase in amine concentration resulted in progressive increase in emission intensity (Fig. 5). The calibration curve for NH_4OH had a linear range at concentration 1–30 mM. The same results were achieved with other small organic amino-containing compounds such as glycine, creatine and adenosine phosphate.

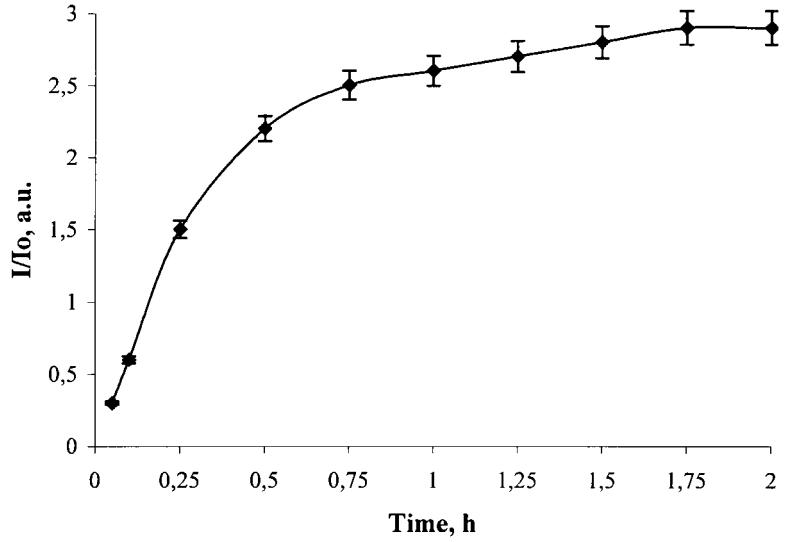

Fig. 3. Kinetics of the isoindole formation in the reaction of the polymer with 23 mM NH_4OH.

The ability of a polymer to bind with protein molecules was tested in the experiments with several proteins. The protein (BSA) addition to polymer suspension also led to a progressive increase in the fluorescent emission similar to NH_4OH (Fig. 6). Due to the large size of protein molecules, the slower kinetics for this reaction in comparison with low-weight organic amines was observed. It was found that the calibration curve for BSA had a linear range at concentration 0.4–1 mM, which is much lower than one observed for NH_4OH. Similar results were obtained with other proteins, such as HRP and haemoglobin. The reason for this lies in the fact that reaction of isoindole formation is partially reversible and dependent on the binding constant which is proportional to the number of interactions between the polymer and analyte. BSA which has 59 lysine and several other active residues binds much more strongly to the polymer than low-weight amines. Due to this, the assay sensitivity for analyte, which contains several amino groups such as proteins and nucleic acids is different (saturation is faster but signal is smaller) in comparison with monoamines.

The binding capacity of the RP was estimated by a BCA method. The protein concentration after sorption was compared with protein concentration before the sorption. It was calculated that 1 g of the polymer can bind 0.6 mg of BSA, 0.55 mg of cytochrome C, 0.2 mg of microperoxidase,

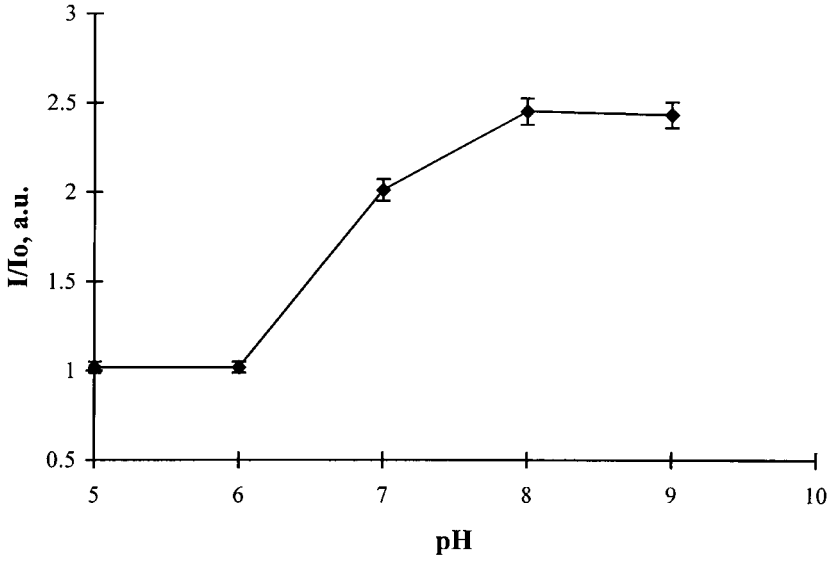

Fig. 4. pH dependence of the isoindole formation and polymer fluorescence.

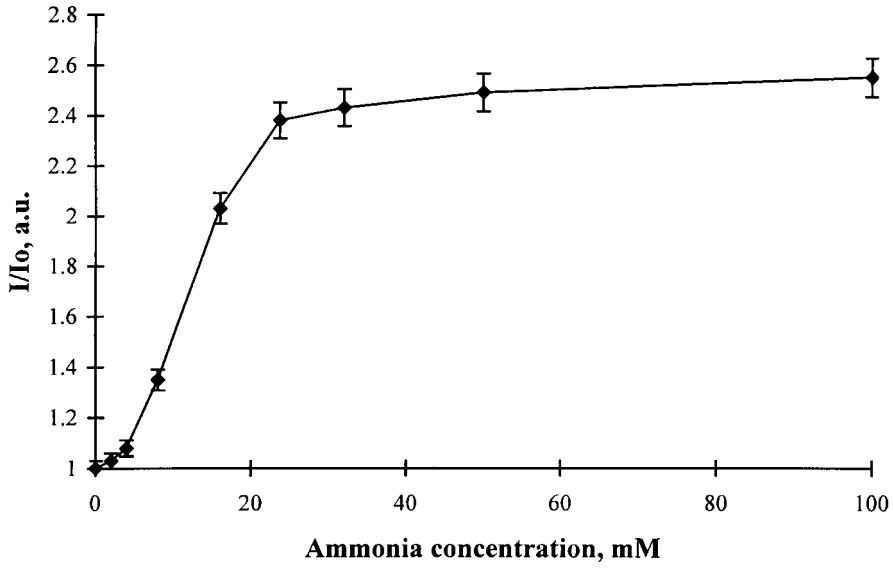

Fig. 5. Change in the emission spectra of the polymer suspension in response to addition of spiked concentration of NH_4OH (concentration 1–100 mM, reaction time — 30 min).

0.5 mg of HRP and 1 mg of haemoglobin (Fig. 7). The binding capacity of the polymer and sensitivity of the fluorescent detection therefore depend on the number of amines available – a function of both the protein's structure and its amino acid composition. The immobilisation rate was found to be comparable with commercial sorbents used for protein immobilisation such as activated CN Sepharose 4B (Pharmacia Biotech, Sweden) [9]. It is anticipated that the polymer can be used as an effective alternative immobilisation matrix in affinity chromatography for immobilisation of low-weight organic amines, proteins and nucleic acids as well as sensor/assay components for primary amine detection. The synthesised polymer is stable and maintains its sorption and detection properties in an oxygen-free environment (degassed solution) at room temperature for six months.

4. Conclusions

A new matrix for immobilisation of the amino-containing substances is proposed. Among the advantages of its application are good immobilisation properties, which do not include the need for pre-activation procedure, loading rates which are comparable with those achieved using the best commercial matrixes and the possibility to evaluate and

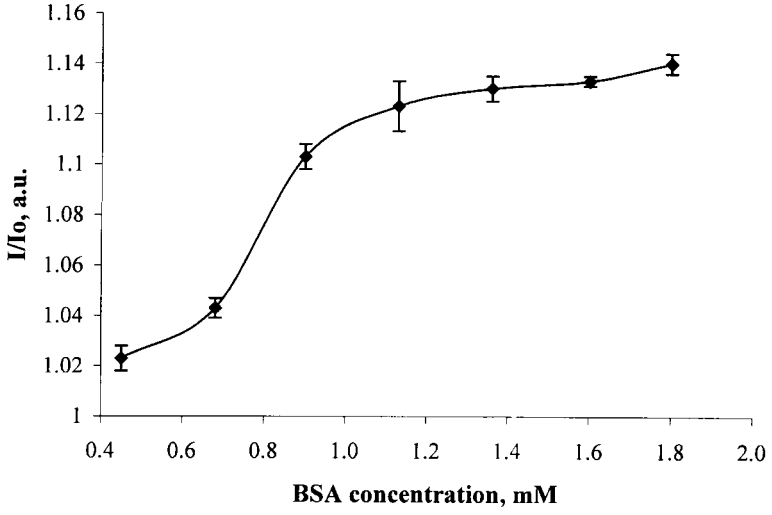

Fig. 6. Change in the emission spectra of the polymer suspension in response to addition of spiked concentration of BSA (concentration 0.4–2 mM, reaction time — 30 min).

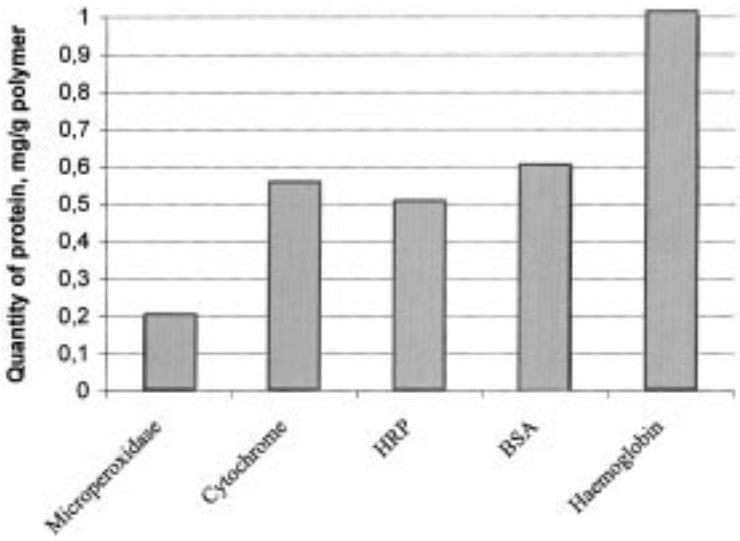

Fig. 7. Binding capacity of RP to different proteins.

detect binding events using fluorescence measurements. Good stability and reasonable sensitivity make the proposed polymer a suitable material for development of chemical sensors with selectivity for primary amines.

References

[1] Galaev IY, Mattiasson B. Trends Biotechnol 1999;17(8):335–40.
[2] Ista LK, PerezLuna VH, Lopez GP. Appl Environ Microbiol 1999;65(4):1603–9.
[3] Whitcombe MJ, Alexander C, Vulfson EN. Trends Food Sci Technol 1997;8(5):140–5.
[4] Simons Jr. S, Johnson DF J Org Chem 1978;43(14):2886–91.
[5] Dai F, Burkert VP, Singh HN, Hinze WL. Microchem J 1997;57(2):166–98.
[6] Piletsky SA, Piletskaya EV, Yano K, Kugimiya A, Elgesma AV, Levi R, Kahlov U, Takeuchi T, Karube I, Panasyuk TI, El'skaya AV. Anal Lett 1996;29(2):157–70.
[7] Piletsky SA, Piletska OV, Weston D, Cullen D, Schedler U, Turner APF. UK Patent application 99092454 (22.04.99).
[8] Osnes T, Sandstad O, Skar V, Osnes M, Kierulf P. Scand J Clin Lab Investig 1993;53(7):757–63.
[9] EAH Sepharose 4B, Manual instructions. Sweden: Pharmacia Biotech, 1998.