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

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Abstract

A new polymer able to react with primary amines was synthesised from allyl mercaptan, o-phtalic dialdehyde and ethylene glycol dimethacrylate by radical polymerisation. Reactive thioacetale formed by allyl mercaptan and dialdehyde can bind primary amino groups without additional pre-activation forming fluorescent isoindole complex. It gives a great opportunity to monitor binding and loading of the amino compounds on 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.

Keywords: amines, immobilisation, polymerisable thioacetale

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Introduction

Recently a development of so-called “smart” polymers has attracted the most notice in biotechnology due to their ability to “sense” the change in the environment or ability to change predictably the environment characteristics. “Smart” polymers are used in bio-separation and drug delivery, for the development of new biocatalists, as biomimetic actuators and as surfaces with switchable hydrophobic-hydrophilic properties [1]. There is a strong interest to “smart” polymers in ecology where environmentally sensitive polymer can control or even prevent the bacterial contamination of the solid surfaces in 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 a synthesis and analysis of new “smart” polymers able to react with primary amines such as ammonia and amino acids, peptides, proteins and nucleic acids. The polymer reactivity is based on well-known reaction between primary amines, o-phtalic dialdehyde (OPA) and β-mercaptoethanol in solution [4]. Homologous aromatic dialdehydes such as o-phthaldialdehyde (OPA, P-2331), naphthalene-2,3-dicarboxaldehyde (NDA, N-1138) and anthracene-2,3-dicarboxaldehyde (ADA, A-1139) are essentially nonfluorescent until reacted with a primary amine in the presence of excess of mercaptan, such as 2-mercaptoethanol, to yield a fluorescent isoindole (Figure 1). The increase in fluorescence emission permitted to monitor the binding events and quantify the concentration of primary amino compounds in solution [5]. Heterogeneous assay based on amino containing polymer and o-phtalic dialdehyde was used to monitor concentration of mercaptoethanol and sialic acid in solution [6].

Despite of high sensitivity achieved in those studies the necessity to use a multicomponent mixture for analysis limits the method application. Recently the synthesis of new polymer, which contains already two out of three components: dialdehyde and mercapto group, necessary for fluorescence complex formation was described [7]. An organic soluble reactive polymer containing thioacetale was prepared as 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 big variety of the monomers, which can be used for tuning of the polymer properties, could bring different practical applications. Here we describe synthesis and investigation of highly cross-linked polymers containing thioacetale. These materials can be used in affinity chromatography for protein and DNA immobilisation and in sensor technology for detection of primary amines.

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. O-Phtalic dialdehyde (OPA), cytochrome C were from Aldrich. Bicinchonic acid (BCA micro reaction kit) was purchased from Pierce. Microperoxidase was bought from Biozyme Laboratories (UK).

2.1. Preparation of the reactive polymer (RP).

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 with size 1-5 μm were collected using seaving and decantation.

2.2. Fluorimetry with reactive polymer.

The typical experiment was performed as follows: 3 mg of RP was 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 isoindole formation the aliquots of concentrated NH_4OH solution was added to polymer solution. A change in fluorescence was recorded as function of time (excitation wavelength Eex=355 nm). All measurements were made in triplicate.

2.3. Protein immobilisation.

The binding capacity of the polymer was demonstrated with several proteins– microperoxidase (1 kD), cytochrome C (12.4 kD), HRP (44 kD), BSA (66 kD) and haemoglobin (67 kD). 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 hours. Protein concentration before and after sorption was measured spectrophotometrically using a BCA method [8] and calculated accordingly to calibration curves made for each protein.

3. Results and discussion

The thioacetale-containing polymer was synthesised as described previously [6]. Its ability to react with primary amines was studied by measuring change in fluorescence upon addition of ammonia hydroxide. Fluorescent measurements were carried out with a

polymer suspension with the size of the particle 1-5 μm, which has a good stability in water. It was found that the addition of the 23 mM of the NH_4OH to the RP suspension increased the excitation and emission intensity in 3 times (Figure 2). The polymer with bound amine had an excitation maximum at 347 nm and an emission maximum at 430 nm. The kinetics of isoindole formation was the same for heterogeneous and homogeneous reaction (Figure 3). The fluorescence emission increased steadily during 2 hours. For practical purposes when faster assay is desirable the signal can be measured in 30 minutes when its magnitude reached 70% of maximum.

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

Progressive increase in amine concentration resulted in progressive increase in emission intensity (Figure 5a). 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 (data not shown).

A polymer ability to bind protein molecules was tested in the experiments with several proteins. The presence of the protein (HRP) in RP suspension also led to progressive increase the fluorescence similar like in case with ammonia hydroxide (Figure 6). Due to 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 limit of saturation observed for ammonia hydroxide. The reason for that lies in

the fact that reaction of isoindole formation is 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 to the polymer much stronger than low-weight amines. Due to this we can explain much higher sensitivity of the assay for analyte able to form multipoint interactions with polymer, such as proteins and nucleic acids.

The binding capacity of the reactive polymer was estimated by BCA method. The protein concentration after sorption was compared with protein concentration before sorption. It was calculated that 1 g of the RP can bind 0.6 mg of the BSA, 0.55 mg cytochrome C, 0.2 mg microperoxidase, 0,5 mg HRP and 1 mg haemoglobin (Figure 6). The binding capacity of the RP and sensitivity of the detection based on utilisation of the RP 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 comparable with commercial sorbents used for protein immobilisation like activated CN Sepharose 4B (Pharmacia, Sweden). It suggests that the polymer can be used as effective alternative immobilisation matrix in affinity chromatography for immobilisation of low-weight organic amines, proteins and nucleic acids.

Accordingly the elemental analysis the allyl mercaptan was included in the polymer for 42% from the theoretical value.

The polymer described is stable and can be stored at room temperature for 6 month without loss of activity.

Conclusions

A new matrix for immobilisation of the amino-containing substances is proposed. Among the advantages of its application are spontaneous immobilisation, high loading and possibility to evaluate and detect binding events using fluorescence measurements. Good stability and reasonable sensitivity make the RP a suitable material for development of chemical sensors with selectivity for primary amines.

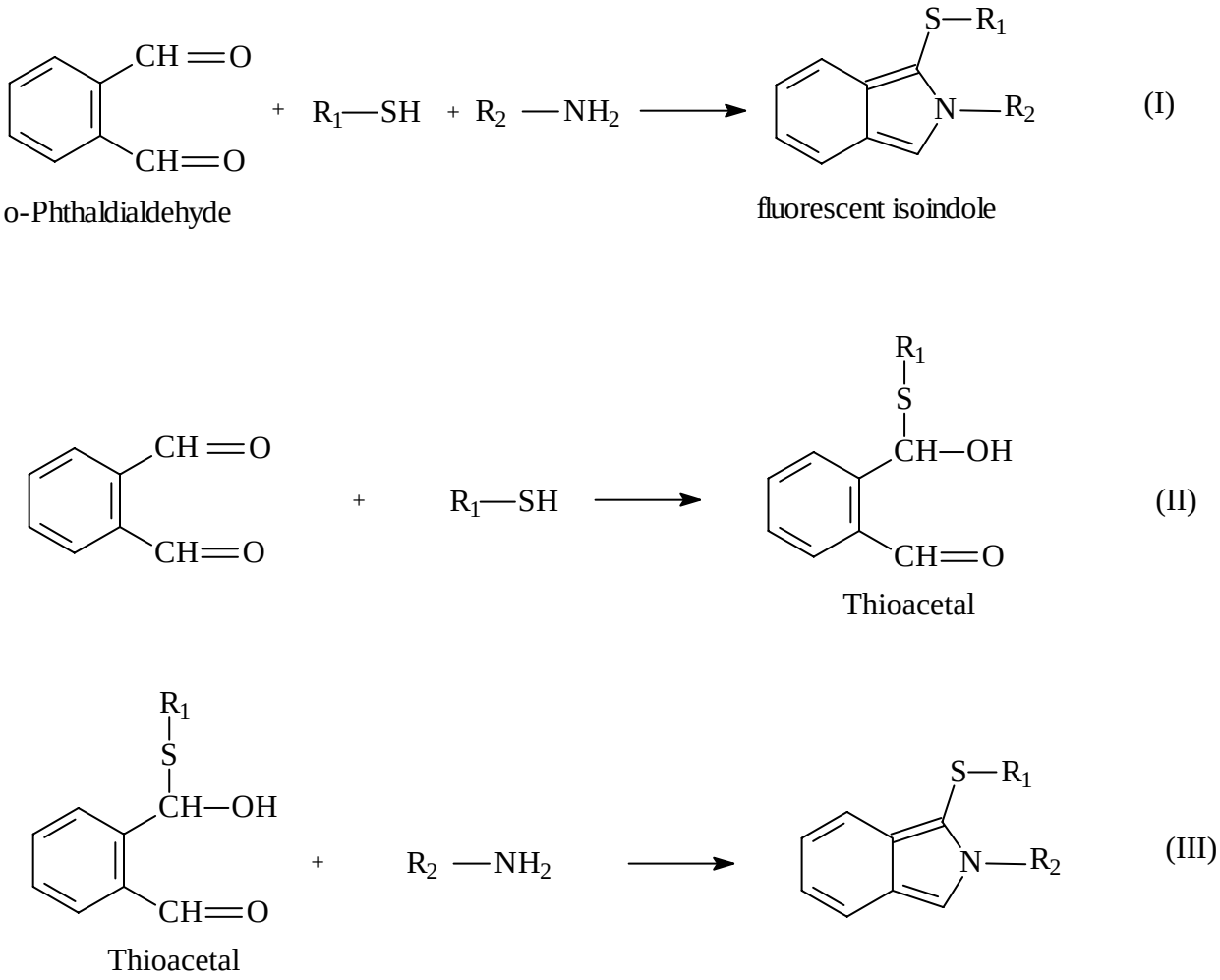

Figure 1. Piletska et al.

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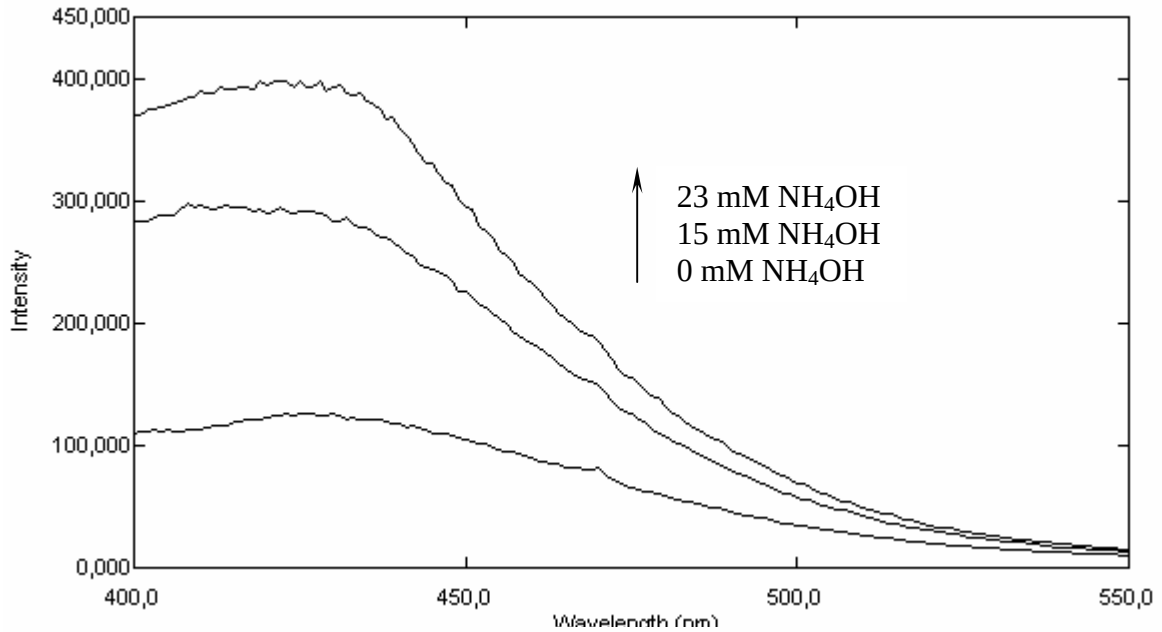

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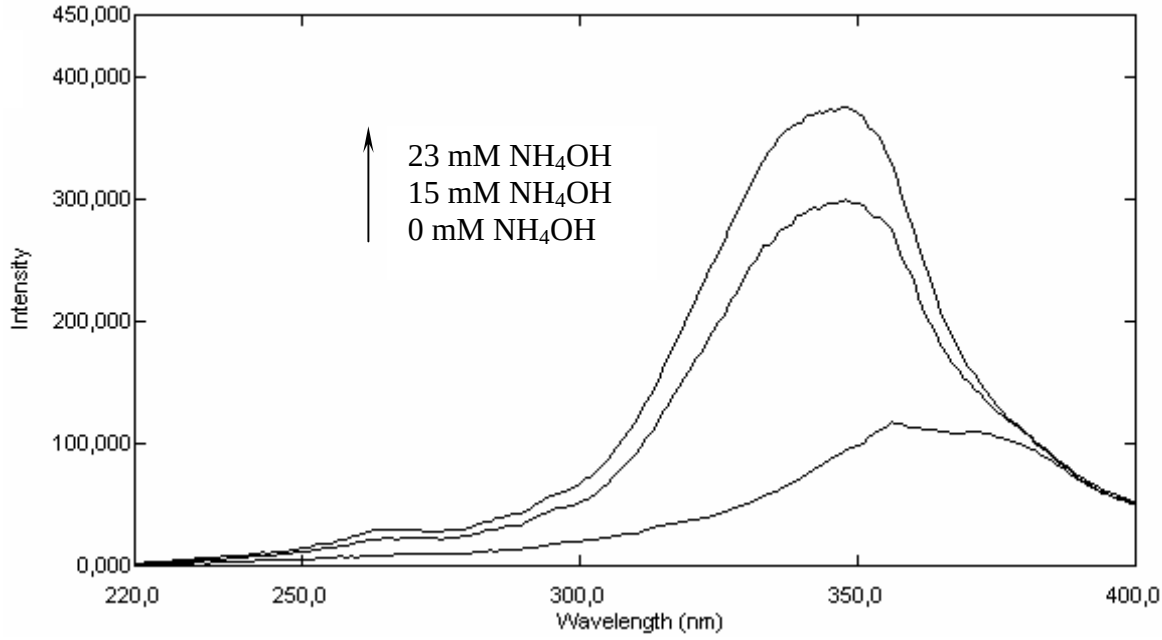
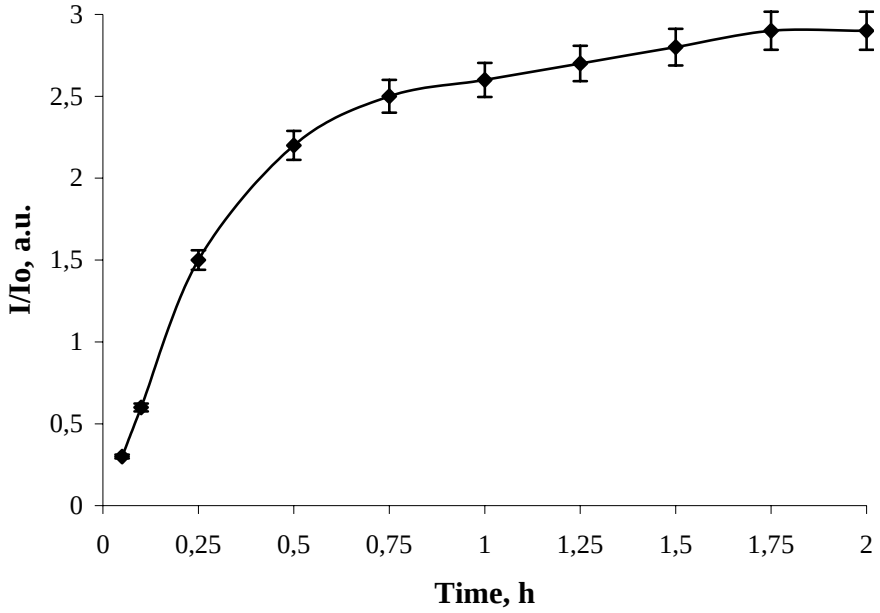


Figure 2. Piletska et al.



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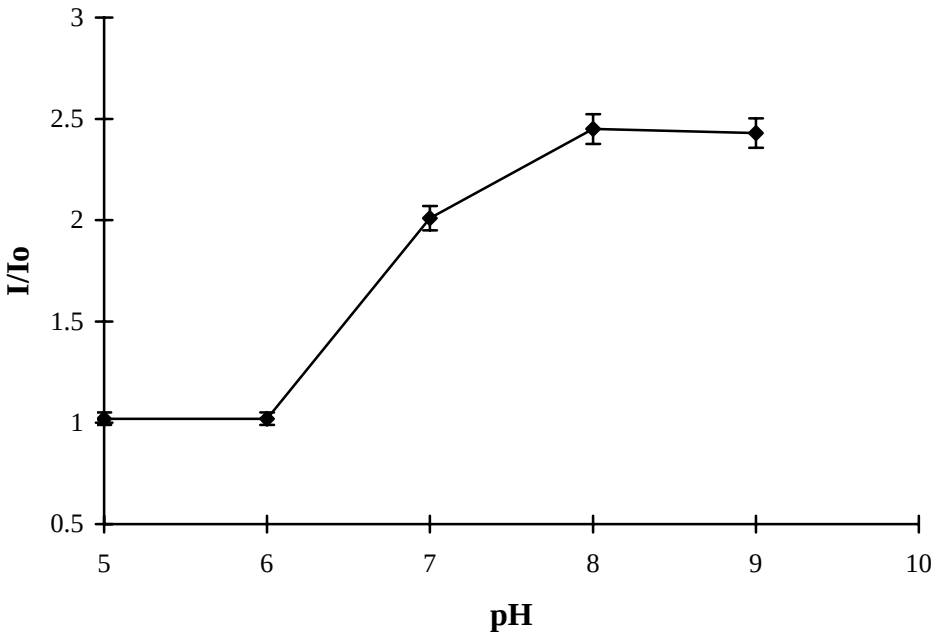

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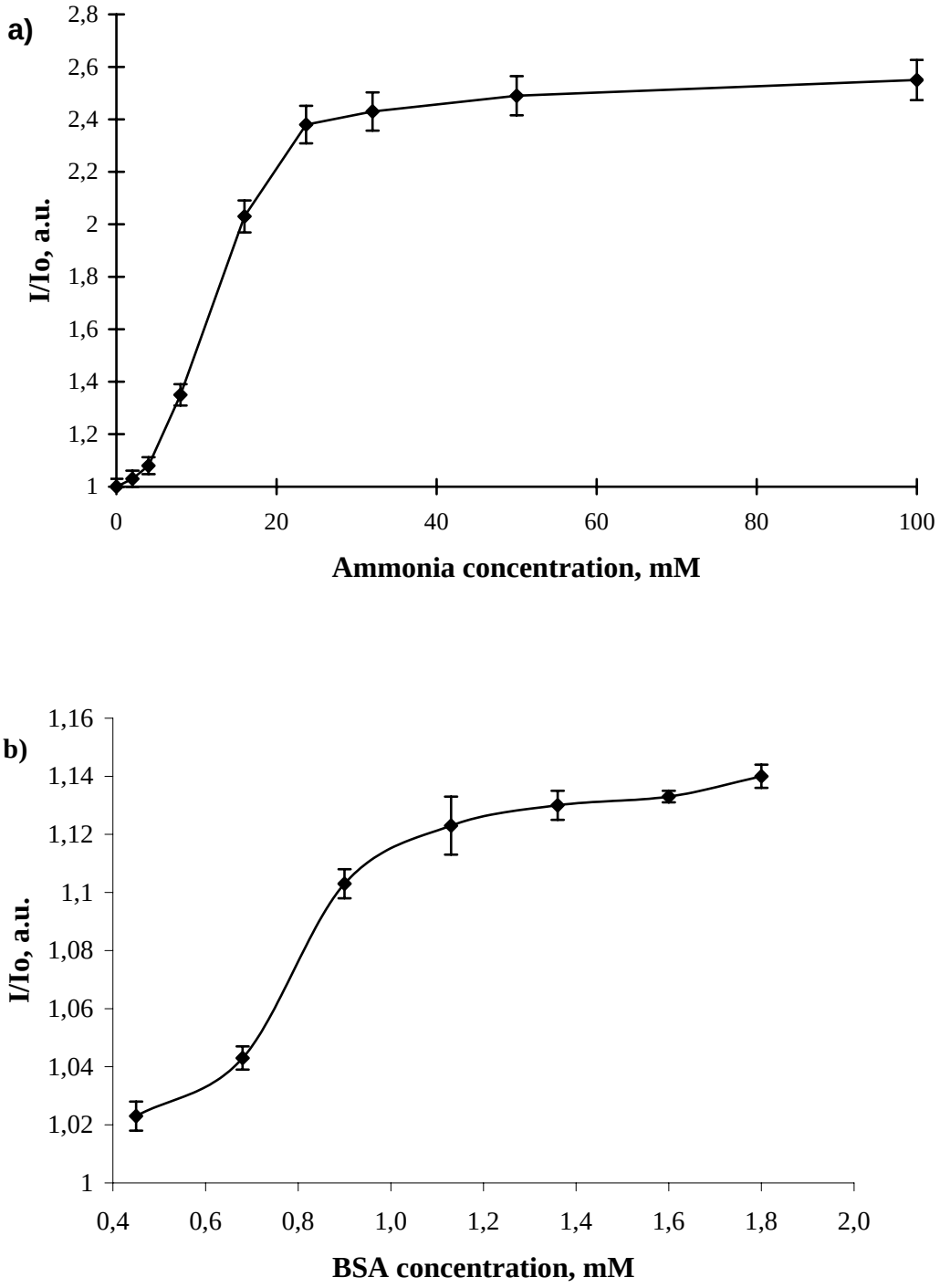

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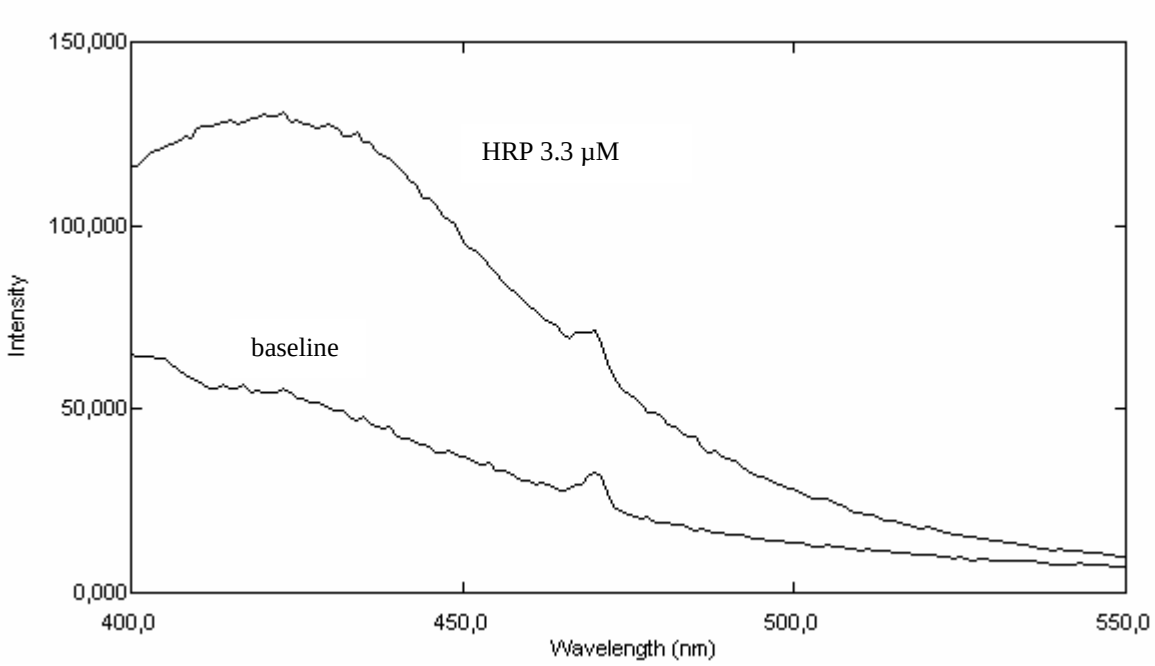

Figure 6. Piletska et al.

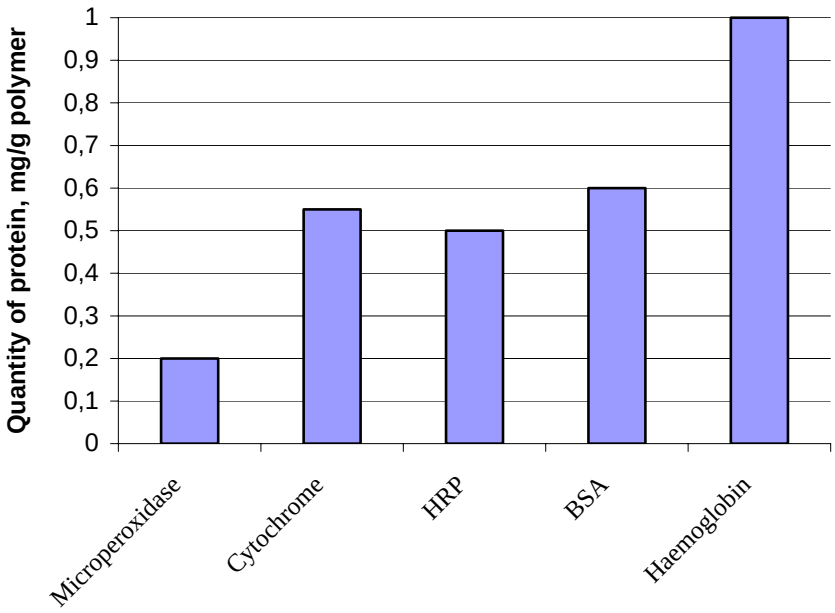

Figure 7. Piletska et al.

Figures captions

Figure 1. Interaction between dialdehyde, mercaptan and primary amine (I), thioacetal formation (II), formation of the fluorescent isoindole complex between thioacetal and primary amine (III). R_1-SH –polymerasable mercaptan (allyl mercaptan), R_2-NH_2 – primary amine.

Figure 2. Changes in the emission (a)) and excitation spectra (b)) of the RP as reaction on presence of ammonia.

Figure 3. Kinetics of the isoindole formation in presence of the 23 mM ammonia.

Figure 4. Dependence of the RP response from pH.

Figure 5. Dependance of the RP emmision spectra for different amino group containing compounds; a) ammonia hydroxide (concentration 1-100 mM, reaction time - 30 min), b) BSA (concentration 0.4-2 mM, reaction time - 30 min).

Figure 6. Changes in the emission intensity of the RP (λ_{exc} 355 nm) as response for binding of HRP (reaction time-18h).

Figure 7. Binding capacity of reactive polymer to different proteins.

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