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Optical responses, permeability and diol-specific reactivity of thin polyacrylamide gels containing immobilized phenylboronic acid

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Abstract

Thin semitransparent gels were prepared by radical copolymerization of *N*-acryloyl-*m*-aminophenylboronic acid (NAAPBA) and acrylamide (AAm) taken in molar ratios from 8:92 to 16:84, respectively, in water. The gels were characterized by the content of immobilized NAAPBA and monomer conversion. Scanning electron microscopy revealed the micrometer size pores in the dried gels. The wet gels displayed a linear optical response to sugars with sensitivity decreasing in the series: D-fructose, D-galactose, D-glucose, D-mannose, *N*-acetyl-D-glucosamine in the sugar concentration range from 1 to 40–60 mM at pH 7.3. Cross-linking of the gels with *N*,*N*-methylene-bis-acrylamide decreased the strength of optical response. Specific binding capacity of a diol-containing dye Alizarin Red S in the gels at pH 7.0 coincided with the content of immobilized NAAPBA indicating the 1:1 stoichiometry of the reaction and, therefore, good accessibility of the boronic acid ligands for water-soluble diols. Permeability of the gels was studied with a non-interacting dye Ethyl Orange exhibiting the pore diffusion coefficient of 1.4×10^{-7} cm^2/s. The rate of optical response of the gels to glucose was found to be determined by diffusion of sugar into the relatively thick gels ($l = 0.35$–1 mm) with effective diffusion coefficients of 2×10^{-7} cm^2/s. In the thinner gels ($l = 0.1$ mm) the input of other kinetic processes, such as affinity binding or structural rearrangements of the gel, was noticeable.

Keywords: Boronic acid; Hydrogels; Sugar sensing

1. Introduction

Optical systems based on responsive hydrogels represent a recent trend in the development of delicate instruments for biomedical studies. In particular, variable-focus liquid microlenses and microlens arrays composed of thermoresponsive gels of *N*-isopropylacrylamide have been recently developed [1]. Optical biosensors containing responsive gels are also of increasing interest [2]. The synthetic approach to selective optical detection of biomolecules involves preparation of hydrogels containing both immobilized receptor with specific affinity to the analyte and optical reporter. This approach has been taken to combine immobilized enzymes, as recognition elements, with pH-sensitive dyes, as optical reporters, in one sensing device [3]. Another class of specific recognition elements is presented by derivatives of phenylboronic acids (PBAs). They are known to bind to 1,2 or 1,3-diols via reversible formation of boronate esters in organic [4] or aqueous solutions [2,5,6]. This kind of affinity binding enables the use of

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PBAs for chemical design of synthetic receptors complementary to water-soluble biomolecules containing diol groups: carbohydrates, vitamins, coenzymes, glycoproteins and ribonucleic acids (RNAs) [5,7–11]. Many of such receptors comprise PBA-containing polymers as well as hydrogels based on such polymers because of their strong, easily detectable responses to the changes in concentration of target molecules. In particular, the PBA-containing gels were employed to create a self-regulated insulin delivery system for the treatment of diabetes, an alternative to common insulin injection [4,5,12]. Binding of sugars to the pendant boronates results in enhanced hydration and conformational changes in the soluble polymers and, hence, in swelling of the boronate-containing hydrogels [13,14]. Several methods have been suggested for the measurements of gel swelling induced by the reactions with saccharides including electrochemical [14] and volumetric [5] detection. Ingenious physical methods for carbohydrate detection have been developed by Asher et al. [2,15] and Lee et al. [13]: crystalline colloid arrays or reflection holograms were incorporated into boronate-containing polyacrylamide gels and diffracted by visible light, whereas the diffraction wavelength reported on hydrogel volume. Lee et al. has shown that the response of the hologram to glucose was proportional to its concentration in the range of 2–11 mM. As recently reported, the change in the peak diffraction wavelength, generated by a holographic sensor, leveled off at ca. 18 mM glucose concentration [15]. Similar results were obtained by Asher et al. [16]: at high concentration of glucose, deformation of the gel was not proportional to sugar concentration, but exhibited a sigmoid dependence in the range from 12 to 120 mM [16]. Although based on highly accurate registration of the diffraction patterns, the above techniques require specific reporters (holograms or photonic crystals). It seems challenging to develop the sugar-specific gels with changing optical properties, which would allow registration of the gel response by a spectrophotometer without auxiliary reporters. The direct optical detection is much easier and might be used as alternative method for the estimation of sugar concentration using boronate-containing hydrogels.

Bioanalytical and biomedical applications of PBA-containing copolymers and gels can be developed further based on the quantitative studies of their structure and properties. Copolymer composition and conversion of monomers into the gel determine both the physical and chemical properties of the responsive material. In particular, its affinity binding capacity depends on the number of functional boronate groups available for the specific binding to the target molecules [6]. Physical material properties such as porosity, transparency and permeability for sugars are important for the strength and the time of the gel response to environmental changes, and, therefore, for the performance of sugar sensors or drug delivery systems. It is generally accepted that it is the charged anionic form of PBA, the boronate anion, which reacts with sugars or diols. It is supposed, therefore, that the affinity of PBAs to sugars increases with pH. However, interactions of neutral form of PBA with *N*-acetylneuraminic acid [17] and Alizarin Red S (ARS) [18] have been reported. It was shown that not only pH, but also many other factors influence the complex formation in the solution, for example buffer composition and steric arrangement of the interacting counterparts [6]. One can expect, therefore, that effects will be exerted on the complex formation by the structure of polymer gel bearing the PBA ligands, including the ligand density, degree of polymer cross-linking, porosity, etc. Most of the existing studies of the responsive boronate-containing materials are lacking quantitative characterization of the amount of immobilized PBA ligands, stoichiometry of the complex formation between the ligands and diols as well as binding capacities of the PBA-containing gels [8,13,16,19]. These characteristics would provide a better understanding of the gel functioning either as a sugar sensor or as a drug deliverer [20].

The rate of response exhibited by a boronate-containing gel to the changing concentration of analyte is another important characteristic determined by the rate of sugar diffusion into the gel as well as by the rate of affinity complex formation and the gel swelling. The diffusion depends on the structure of the gel network; the large pores seem to be preferential for a fast transport of analyte [21] and therefore, for a fast response. To the best of our knowledge, porosity of PBA-containing gels has not yet been considered in the literature. The apparent diffusion coefficients of sugars into boronate-containing gels were rarely reported whereas the available calculations were based upon the assumption of direct proportionality between the potentiometric response of the gel and the amount of bound sugar [22], which needs further studies.

The aims of the study are to show the possibility of optical detection of sugars using PBA-containing gels and to characterize quantitatively their ligand densities, affinity binding capacities and permeabilities for organic molecules, and also to evaluate the roles of different kinetic processes in the overall rate of the gel response to sugars and to obtain the quantitative parameters of sugar diffusion into the PBA-containing gels. We have synthesized and characterized a series of semitransparent gels composed of copolymers of acrylamide (AAm) and *N*-acryloyl-*m*-aminophenylboronic acid (NAAPBA) changing their transparency in response to the varied concentration of sugars in the containing solutions. The roles of PBA ligand density and the presence of bifunctional cross-linker for the optical response of the gels have been investigated.

2. Experimental

2.1. Materials

Acrylamide (AAm), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulphate, D-(+)-glucose, *N*-acetyl-D-glucosamine, *N*,*N*′-methylene-bis-acrylamide (BisAAm) and alizarin sodium monosulfonate (Alizarin Red S, ARS) were products of Sigma Aldrich (Steinheim, Germany). *N*-Acryloyl-*m*-aminophenylboronic acid (NAAPBA) was prepared as described elsewhere [23]. Sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate anhydrous, sodium bicarbonate, sodium chloride, sodium hydroxide and D-(+) galactose were purchased from Merck (Darmstadt,

Germany). Ethyl Orange, α-lactose and D-(−)-fructose were products from BDH Chemicals Ltd (Poole, England). Sepharose CL-6B was purchased from Amersham Pharmacia Biotech AB, (Uppsala, Sweden). Potassium sodium tartrate tetrahydrate was a product of Fluka (France). 3,5-Dinitrosalicylic acid was a product of Aldrich Chemical Company, Inc. (Milwaukee, USA). D-(+)-Mannose was purchased from Fluka (Buchs, Switzerland). 3-Acrylamidopropyltrimethoxy silane (3-AAPTMS) was a kind gift from Dr. J. Ekeroth (Eka Chemicals AB, Bohus, Sweden).

2.2. Preparation of AAm–NAAPBA gel on a glass slide surface

NAAPBA (36, 42, 48 or 84 mg) and AAm (156 mg, 2.2 mmol) were dissolved in distilled water (2.5 mL) at 50 °C, the reaction mixture contained 7.9, 9.1, 10.2 or 16.7 mol% of NAAPBA, respectively. TEMED (10 μL) was added to the solution and the reaction mixture was degassed. Glass slides (36 mm × 9 mm) were cleaned with detergent, rinsed subsequently with distilled water and with aqueous solution of Tween 20 (0.1% v/v) and dried in air. The reaction mixture (200 μL) was mixed with a freshly prepared ammonium persulphate solution (100 μL, 40 mg/mL) on the surface of a glass slide. The white copolymer gel formed immediately after mixing of the solutions. The gel-coated glass slides were kept in a desiccator over distilled water for 2 h. After that, the gels were either stored in distilled water or assayed for the fraction of non-reacted NAAPBA. For preparation of thinner gels the volumes of mixed solutions were proportionally reduced (100 + 50 μL, etc.).

2.3. Preparation of AAm–NAAPBA–BisAAm gel on a glass slide surface

The glass plates (36 mm × 9 mm) were successively treated with 4 M NaOH (2 days), distilled water, 4 M HCl (2 days) at room temperature (22 °C), washed with distilled water, ethanol, dried under vacuum and placed into 3-AAPTMS solution (5% v/v) in 1,4-dioxane. The chemical modification was carried out in a boiling solution of 3-AAPTMS for ca. 16 h. Then, the glasses were rinsed with fresh 1,4-dioxane and dried under vacuum in a desiccator. NAAPBA (48 mg, 0.25 mmol), AAm (156 mg, 2.2 mmol) and BisAAm (15.6 mg, 0.1 mmol) were dissolved in distilled water (2.5 mL) at 50 °C. TEMED (10 μL) was added to the solution. AAm–NAAPBA–BisAAm gels formed immediately after mixing of the solutions on the chemically-modified glass surface.

2.4. Estimation of NAAPBA conversion into the gel

AAm–NAAPBA gel was prepared as described above. The conversion of NAAPBA was estimated by means of thorough extraction of non-bound NAAPBA from the gel followed by NAAPBA assay in the extract. The glass slide was placed into a cuvette containing 3 mL of 0.1 M sodium phosphate buffer, pH 7.0. Washing was carried out on a rocking table for 24 h. UV measurements of the washings were made in the wavelength range from 200 to 350 nm, after the first 30 min, 1 day and 2 days of the extraction, each time with a fresh buffer solution, indicating that more than 99.5% of the extractable monomers and initiators were removed from the gel during 1 day extraction. The amount of washed out NAAPBA was estimated spectrophotometrically at $\lambda_{max} = 263$ nm after separation of NAAPBA from the non-reacted admixtures of TEMED, AAm and ammonium persulphate by adsorption chromatography on Sepharose CL-6B as described previously [24]. Briefly, a glass column (1 cm × 12 cm) packed with Sepharose CL-6B was equilibrated with 0.1 M sodium bicarbonate buffer, at pH 9.2. The extract was diluted 2-fold by the buffer and applied (0.5 mL) to the column. Elution was performed with the above buffer solution at a flow rate of 0.3 mL/min, at room temperature, absorbance in the fractions (1 mL) being measured at 263 nm. NAAPBA was retained on the column ($V_{el} = 25$ mL) whereas the admixtures were eluted near the total column volume ($V_t = 10$ mL). The amount of the extracted NAAPBA was calculated using the extinction coefficient ($\varepsilon = 9800\ M^{-1}\ cm^{-1}$) estimated for NAAPBA in the same buffer solution.

2.5. Estimation of the gel thickness

AAm–NAAPBA gel was prepared as described above (see Section 2.2). Thickness of the gel (l) was determined by:

$$l = \frac{m}{Sd} \tag{1}$$

where m is the gel weight, d is the gel density and S is the gel area. For estimation of the area each glass slide was divided by a scalpel into $n = 5–6$ rectangular sections, depending on the shape of the gel. The length (L_i) and the width (W_i) of each section were measured by a ruler and the surface area of the gel was calculated by

$$S = \sum_{i=1}^{n} W_i L_i \tag{2}$$

The glass slide was weighed on analytical balance, the gel was scratched out of the glass slide using a spatula and the glass slide was weighted again. The difference in the glass slide weights before and after the gel scratching and the scratched gel weight were similar, which were averaged to produce m. Since the main component of the copolymer gel is water (>92%) and the density of polyacrylamide is 1.13 g/cm^3, the density of the gel was assumed to be 1.0 g/cm^3. The gel thickness l calculated as given above is an average value for each type of gels and is listed in Table 1. The electron scanning microscopy images of the cracks in a single dry gel show that the variations in the gel thickness are not large and the gel has almost the same thickness on the most part of its area (data not shown).

Table 1
Characteristics of AAm–NAAPBA gels of different thicknesses and apparent diffusion coefficients of glucose into the gels[a]

Group of gels	Monomer mixture (μL)	Ammonium persulphate (40 mg/mL) (μL)	Surface area (cm^2)	Weight (g)	Thickness (mm)	Apparent diffusion coefficient of glucose (cm^2/s)
1	200	100	2.7 ± 0.1	0.27 ± 0.06	1.0 ± 0.2	$(2.9 \pm 1.3) \times 10^{-7}$[b]
2	150	75	2.56 ± 0.2	0.21 ± 0.02	0.83 ± 0.1	$(2.1 \pm 0.4) \times 10^{-7}$[b]
3	100	50	1.95 ± 0.1	0.07 ± 0.01	0.35 ± 0.07	$(2.6 \pm 1.0) \times 10^{-7}$[b]
4	50	25	1.3 ± 0.07	0.012 ± 0.003	0.13 ± 0.08	$(5.3 \pm 0.3) \times 10^{-8}$[c]

The errors are standard deviations obtained by measurements of three or four independently prepared gels.
[a] Molar percentage of NAAPBA in the monomer mixture was 10.2%.
[b] Calculated from Eq. (3).
[c] Calculated from Eq. (5).

2.6. Sorption and desorption of Alizarin Red S (ARS) and Ethyl Orange (EO)

ARS or EO was dissolved at 4.5 mM concentration in 0.1 M sodium phosphate buffer solution, pH 7.0. The gel-coated glass slide was placed into a cuvette, containing 4 mL of the freshly prepared ARS or EO solution. The gels were incubated in the solutions of dyes at room temperature on a rocking table for the time intervals from 5 min to 20 h. After staining, the colored gels were repeatedly washed with 3 mL portions of the above buffer solution on a rocking table. The solution was replaced with a fresh portion every 5 min. The absorbance of the colored washings was monitored at $\lambda_{max} = 500$ nm for ARS and at $\lambda_{max} = 475$ nm for EO. The procedure was continued until the constant optical density of the washings has been reached. To elute the specifically adsorbed ARS from the gel, the above buffer solution containing 0.5 M fructose was used. The elution was performed by means of the gel incubation with the fructose solution on the rocking table, the eluent being replaced with a fresh portion for each 5 min during the first hour and at longer time intervals later on. The elution of ARS and absorbance measurements of the colored eluate ($\lambda_{max} = 500$ nm) were carried out until the colorless state of the gel had been attained. The absorbances were used for calculation of the amounts (μmol) of the washed out and/or eluted dyes using the calibration curves, obtained for ARS and EO ($\varepsilon_{500(ARS)} = 4300\ M^{-1}\,cm^{-1}$, $\varepsilon_{475(EO)} = 28\,000\ M^{-1}\,cm^{-1}$).

2.7. Optical responses of AAm–NAAPBA gels to sugars and glucose depletion benefice

A gel-coated glass slide was equilibrated with 50 mM sodium phosphate buffer solution (3.4 mL) at given pH in a spectrophotometric cuvette. Optical density of the gel was monitored at 500 nm to generate the baseline. One molar solution of saccharide (3.5–175 μL) in the buffer solution was added to the cuvette and diluted to the total volume of 3.5 mL in order to get desired concentration of the saccharide in the range of 1–50 mM. The optical density was recorded with 5 or 10 min intervals, depending on the rate of the reaction. As soon as a constant optical density has been reached, the glucose solution in the cuvette was replaced with 3.5 mL of the buffer solution and recording of the gel optical density was continued. For measurements of glucose depletion kinetics, several probes (0.1 mL) of the contacting solution were taken out from the cuvette during the depletion of glucose from the gel. These samples were analyzed for glucose using 3,5-dinitrosalicylic acid (DNS) assay [25], see Section 2.8.

2.8. Glucose assay

DNS method was used for determination of glucose concentration [25]. DNS reagent was prepared by dissolving 3,5-dinitrosalicylic acid (1 g), potassium sodium tartrate (30 g), 2 M NaOH solution (20 mL) in distilled H_2O (50 mL). DNS reagent (0.5 mL) was added into a test tube containing 0.1 mL of the glucose sample and 0.4 mL of distilled water. The mixture was heated at 95°C for 5 min. After cooling to room temperature, the optical density of the mixture was recorded at $\lambda_{max} = 540$ nm. Calibration curve was obtained by using glucose solutions with known concentration in the range of 100 μM–10 mM ($\varepsilon_{540} = 107\ M^{-1}\,cm^{-1}$) [25].

2.9. Estimation of apparent diffusion coefficients of glucose into the gels

The AAm–NAAPBA gels with different thicknesses were prepared on glass plates (see Section 2.2 and Table 1) using 10.2 mol% concentration of NAAPBA in the monomer mixture. Optical density changes (ΔOD) of the gels were detected as described in Section 2.7 with 40 mM glucose in 50 mM sodium phosphate buffer solution, pH 7.3. Apparent diffusion coefficients were calculated from the rate of the OD changes by using two different diffusion models and assuming that the measured ΔOD was proportional to the glucose mass uptake. The validity of this assumption is discussed in Section 3.3.

2.9.1. Model 1

In this model, diffusion coefficient (D) can be calculated using Eq. (3), which describes the initial rate of sorption into a thin plane gel under conditions where the system is far from equilibrium [26,27]. This model can be used for low F values exhibiting linear dependence from $t^{1/2}$. This model turned out to be suitable for thick AAm–NAAPBA gels (groups 1–3, see Table 1).

F was plotted against $t^{1/2}$ and D was calculated from the slope, see Eq. (4).

$$F = \frac{\Delta OD_t}{\Delta OD_\infty} = \frac{4}{l}\left(\frac{Dt}{\pi}\right)^{1/2} \quad (3)$$

$$\text{Slope} = \frac{4}{l}\left(\frac{D}{\pi}\right)^{1/2} \quad (4)$$

where F – fractional attainment of equilibrium, ΔOD_t – the optical density at time t, ΔOD_∞ – the optical density at equilibrium, l – the thickness of the gel (as described in Section 2.5), D – the diffusion coefficient of substance.

2.9.2. Model 2

This model (see Eq. (5)) describes the late phase of a sorption process into a thin plane gel, where diffusion is rapid and its initial rate cannot be reliably measured [26,27]. In this case the measurable F values are high and there is no linear dependence of F on $t^{1/2}$. This model was chosen for the calculation of D in thin gels (group 4, see Table 1).

By rearranging Eq. (5) we got Eq. (6). The linear dependence of $\ln(1-F)$ on t was plotted and D was calculated from the slope, see Eq. (7).

$$F = \frac{\Delta OD_t}{\Delta OD_\infty} = 1 - \frac{8}{\pi^2}\, e^{-\pi^2(Dt/l^2)} \quad (5)$$

$$\ln(1-F) = \ln\left(\frac{8}{\pi^2}\right) - \left(\frac{\pi^2 D}{l^2}\right)t \quad (6)$$

$$\text{Slope} = \left(\frac{\pi^2 D}{l^2}\right) \quad (7)$$

where F – fractional attainment of equilibrium, ΔOD_t – the optical density at time t, ΔOD_∞ – the optical density at equilibrium, l – the thickness of the gel (as described in Section 2.5), D – the diffusion coefficient of substance.

2.10. Study of AAm–NAAPBA gel permeability for Ethyl Orange (EO)

AAm–NAAPBA gel of 0.13 mm thickness with 10.2 mol% concentration of NAAPBA in the monomer mixture was prepared as described in Section 2.2. The gel was incubated in 3 mL of freshly prepared 2.2 mM EO solution (50 mM sodium phosphate buffer, pH 7.3) for 4 h on a rocking table. The colored gel was placed into a spectrophotometric cuvette containing 50 mM sodium phosphate buffer, pH 7.3 (3 mL), along the wall parallel to the optical path. Optical density of the solution containing the desorbed dye was monitored at $\lambda_{max} = 475$ nm with 30 s time intervals under gentle magnetic stirring. The absorbances were used for calculation of the amount of the desorbed dye using $\varepsilon_{475} = 28\,000\ M^{-1}\,cm^{-1}$. The diffusion coefficient was calculated as described in Section 2.9 using Model 2. All the experiments described in the Sections 2.6, 2.7, 2.9 and 2.10 were performed at room temperature (22 °C).

2.11. Preparation of dry gels for electron microscopy

AAm–NAAPBA gels prepared as described above were dried in two different ways. The first method involved drying by aqueous ethanol and the second was freeze drying.

Drying by ethanol. The gels were incubated in aqueous ethanol solutions with gradually increasing ethanol concentration (20, 50, 70 and 80%) at least 30 min in each. After that the samples were dried at room temperature under vacuum for 2 h.

Freeze drying. Inner walls of the bottles for freeze drying (50 mL) were coated by thin layer of ice by freezing distilled water (1 g) on solid carbon dioxide (dry ice). The glass slides with gels were placed into the bottles and frozen on dry ice. The samples were kept overnight at −80 °C and after that freeze dried. The glass slides covered with dried gels were fixed on the stubs, coated with palladium and examined in a Philips XL20 scanning electron microscope.

3. Results and discussion

3.1. Optical responses of the gels to glucose

Semitransparent hydrogels were prepared by radical copolymerization of AAm and NAAPBA on a glass slide surface as described in Section 2.2 (see Fig. 1). The AAm–NAAPBA gels were formed without any bifunctional cross-linker as a result of physical cross-linking during polymerization. Physical properties of the gels are summarised in Table 1. Conversions of monomeric AAm and NAAPBA into the gels of group 4 (see Table 1) were ca. 55 and 40%, respectively. Average PBA ligand density in the gels was 160 ± 10 μmol/mL gel, at the molar ratio of monomers 10:90. Scanning electron microscopy revealed the micrometer size pores in the dried gels. The porous structures resulted from the phase separation occurred during the copolymerization (see Fig. 2).

Due to the scattering of incident light the prepared colorless semitransparent hydrogels exhibited high optical densities at 500 nm. The pH of the buffer and the presence of sugars strongly influenced the optical density values. The values decreased with increasing pH and these changes were dramatically amplified by the addition of glucose (see Fig. 3). The results were well reproducible and indicated the principal possibility of glucose detection in the pH range from 6.5 to 8.0. As the pK_a of boronate groups in soluble NAAPBA–AAm copolymer is about 9 [24], PBA groups in the gel are present in both neutral and charged states under these conditions. Turbidity of the gel most probably originated from the association of the polymer chains due to hydrophobic interactions between

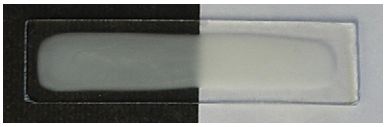

Fig. 1. A photograph of AAm–NAAPBA gel prepared on a glass slide (9 mm × 36 mm), on a black and white background. Gel thickness is 0.13 mm.

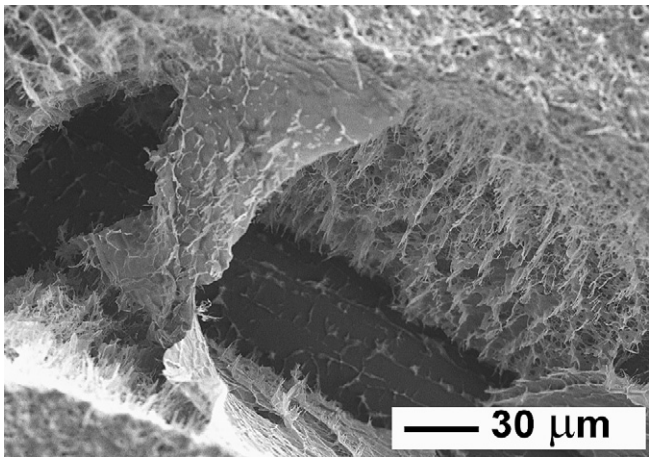

Fig. 2. Electron microscopic photograph of an AAm–NAAPBA gel formed on the glass surface and dried by aqueous ethanol, see Section 2.11.

the neutral PBA groups, resulting in the gel microheterogeneity. Accordingly, the optical density was lower at higher pH where the PBA groups were negatively charged and the gel was better hydrated. Further, the complex formation between the boronates and sugars shifted the pK_a of the PBA groups to lower values [12], increased hydrophilicity of the polymer chains, decreased their association, and, therefore, the microheterogeneity of gels. The increase in pH resulted in increasing fraction of the ionized PBA groups [24] capable of interaction with sugars; therefore sensitivity of the gel to sugar became higher at higher pH. The process of sugar-induced turbidity changes was completely reversible, i.e. the original optical density of the gel at a given pH was restored after incubation of the gel in the sugar-free buffer (see Fig. 4). An AAm–NAAPBA gel could be used as a sugar responsive material daily for at least 1 month without changes in sensitivity and allowed to perform many tens of cycles of the sugar absorption and depletion.

We have compared the glucose sensitivities of physically and chemically cross-linked gels. For this purpose AAm–

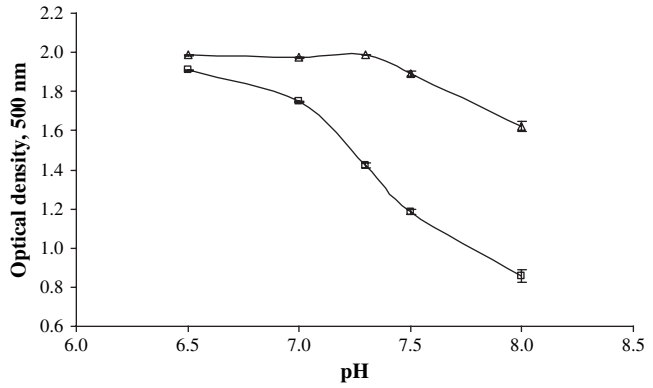

Fig. 3. Optical density of AAm–NAAPBA gel in 50 mM sodium phosphate buffer solution at different pH with (□) and without (△) 40 mM glucose. The error bars indicate the standard deviation of three measurements. Thickness of the gel is 0.35 mm.

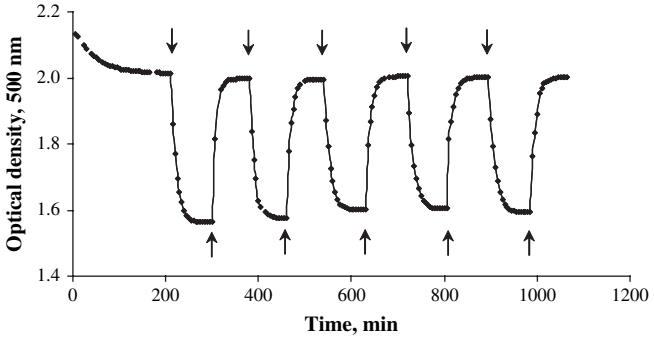

Fig. 4. Optical responses of AAm–NAAPBA gel to 30 mM glucose in 50 mM sodium phosphate buffer solution, pH 7.3. Gel thickness is 0.35 mm. The downward arrows indicate the immersion of the gel into the glucose solution and the upward arrows indicate the immersion of the gel into the buffer solution.

NAAPBA gel cross-linked with *N*,*N*′-methylene-bis-acrylamide (BisAAm) (4 mol% of the monomer mixture) was prepared on the glass surface chemically modified with organosilane containing acrylamide group (see Section 2.3). Both gels exhibited optical responses to the changing glucose concentration in the contacting buffer solution, see Fig. 5. Obviously, the response of the cross-linked gel to glucose was ca. 2-fold weaker then that of the gel without chemical cross-linker (see Fig. 5). It is known that in any functionalized gel, swelling response to a specific stimulus can be regulated by changing the concentration of the environmentally responsive functional groups and/or the cross-link density [16,28]. The elastic resistance of the gel to swelling increases with increasing of the cross-linker content [28]. As the swelling process was limited by the chemical cross-linking, possibility of changes in microheterogeneity and, therefore, turbidity of the gel, was limited as well. It is relevant to note that the background turbidity of the chemically cross-linked gels was much higher than that of the physically cross-linked gels (see Fig. 5a and b). The high gel turbidity may result in scattered baselines and, therefore, in a reduced gel sensitivity to low concentrations of sugars. The time of response to glucose and reproducibility of the optical density changes were very similar for both the gel types, as follows from Fig. 5. Therefore, the chemically cross-linked gels did not display any advantages in sensitivity and/or rate of their response to glucose. Recently, Asher et al. reported the possibility to decrease detection sugar limit of the boronic acid-containing sensor by decreasing the hydrogel cross-linking [16]. These findings agree well with our results.

To improve further the response of the gel to sugars it might be reasonable to increase their boronate ligand density. We have compared the AAm–NAAPBA gels with the NAAPBA concentration of 10.2 and 16.7 mol% in the monomer mixture. The higher concentration of NAAPBA resulted in the increased gel turbidity and microheterogeneity probably caused by the enhanced hydrophobic interactions between the neutral PBA groups in the zones enriched in PBA ligands, see Fig. 6 and Table 2. A similar effect of the ligand content on the copolymer solubility was observed in our previous study [23]: AAm–NAAPBA copolymer with high content of NAAPBA

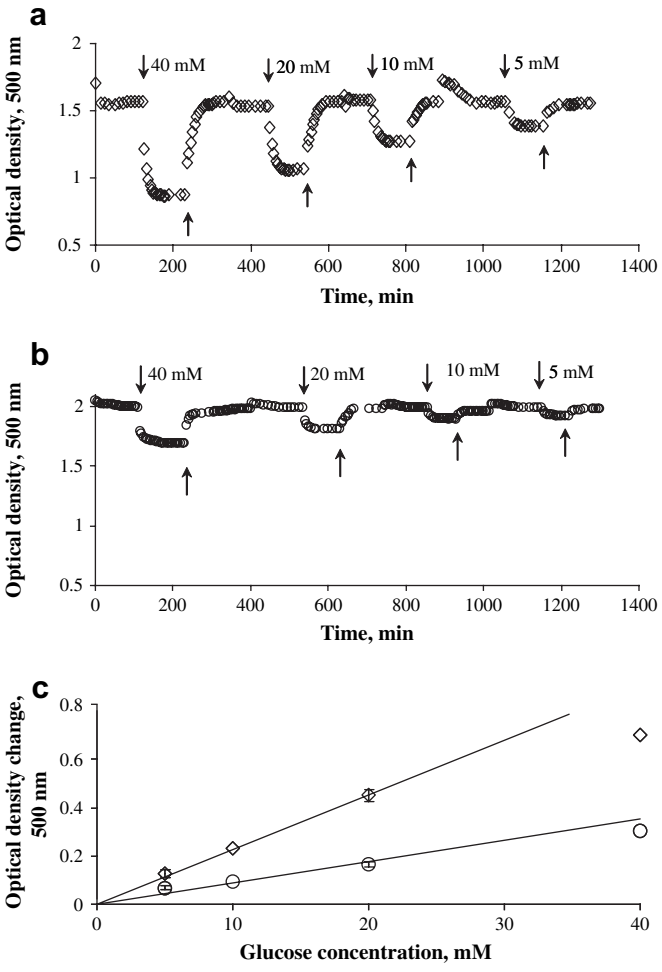

Fig. 5. Optical responses of AAm–NAAPBA (◇) and AAm–NAAPBA–BisAAm (○) gels to different concentrations of glucose in 50 mM sodium phosphate buffer solution, pH 7.5. The downward arrows indicate the immersion of the gel into glucose solution of the indicated concentrations and the upward arrows indicate the immersion of the gel into the buffer solution. The error bars indicate the standard deviation of three measurements. Thickness of the gel is 0.13 mm.

(13 mol%) formed insoluble aggregates at pH < 8.0, whereas the fraction of the copolymer with lower content of PBA (9 mol%) remained soluble. Interestingly, the gel containing less NAAPBA reacted with the glucose solution much faster, and the optical response was ca. 1.2-fold stronger compared to that of the gel with the high ligand density, see Fig. 6 and Table 2. The faster responses of the weakly cross-linked gels have been recently reported by Ben-Moshe et al. [2]. Lee et al. observed the maximum swelling response of PBA-containing polyacrylamide gel to glucose at an initial NAAPBA concentration of 20 mol% in the polymerization mixture [13]. Above this concentration, the sensor became less responsive, probably due to hydrophobic associations within the polymer and increase in the polymer rigidity, as explained by the authors. The rigid gel structure with high ligand density slowed down the swelling process. Thus, the decreased ligand content could improve the gel sensitivity (see Table 2). We have prepared gels with molar percentage of NAAPBA of

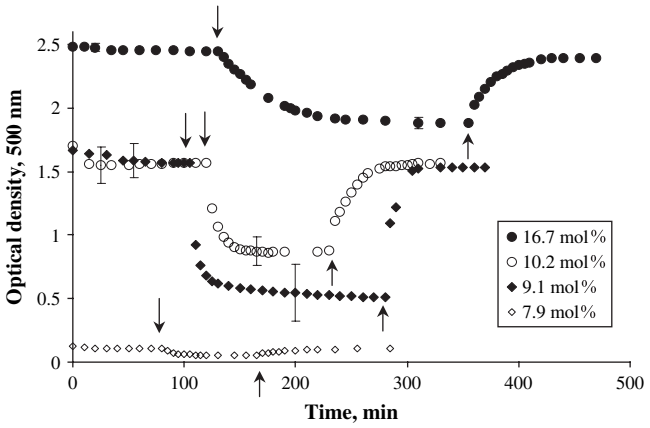

Fig. 6. Optical responses of AAm–NAAPBA gels with different NAAPBA concentration in initial monomer mixture to 40 mM glucose solution in 50 mM sodium phosphate buffer solution, pH 7.5. The downward arrows indicate the immersion of the gel into glucose solution and the upward arrows indicate the immersion of the gel into the buffer solution. The error bars indicate the standard deviation of three independent gels. Thickness of the gels is 0.13 mm.

7.9 and 9.1% in the initial monomer solution. The gel with the lowest NAAPBA molar percentage was almost transparent and could not give any good optical response (see Fig. 6). Hence, too low immobilized ligand density happened to be disadvantageous. On the other hand, the AAm–NAAPBA gel prepared at NAAPBA concentration of 9.1 mol% had almost the same initial optical density as that of concentration 10.2 mol%, but the response to the glucose solution was 1.5-fold faster and 1.3-fold stronger for the former gel (see Fig. 6). It seems that the gels prepared at NAAPBA concentration of 9.1 mol% exhibited nearly optimal characteristics for sugar detection. However, the range of acceptable NAAPBA molar percentage has happened to be relatively narrow so that the above gels were less reproducible compared to the others, as illustrated by the error bars in Fig. 6. In addition, the optical density of the gel saturated by glucose was not completely stable. This was the reason why the gels prepared at 10.2 mol% of NAAPBA were chosen for the further studies.

3.2. Specific affinity binding capacity of the gels

Alizarin Red S (ARS) fluorescent dye was used to study specific reactivity of the AAm–NAAPBA gels toward diols and to quantify the amount of accessible PBA ligands. Having

Table 2
Characteristics of AAm–NAAPBA gels prepared with different NAAPBA content in the monomer solution[a]

NAAPBA content in monomer solution (mol%)	Optical density of the gel at 500 nm	Binding capacity (μmol/mL)
7.9	0.12 ± 0.03	ND
9.1	1.57 ± 0.13	ND
10.2	1.56 ± 0.15	160
16.7	2.48 ± 0.04	320

ND – not determined; the errors are standard deviations obtained by measurements of three or four independently prepared gels.
[a] Thickness of the gels is 0.13 mm (group 4, Table 1).

a reactive diol group, ARS is able to form a complex with PBA at pH 7.0 [18]. The boronate ester formation changes both the absorbance and fluorescence spectra of the dye. The complex formation can be easily detected by the naked eye: a dark red color of aqueous ARS solution at pH 7.0 turns into a bright orange-yellow when the dye is absorbed by AAm–NAAPBA gel. The addition of a carbohydrate into the PBA–ARS system sets up a new equilibrium between carbohydrate and boronic acid by displacing of ARS [18]. We have found that the absorbed ARS could be completely desorbed from the AAm–NAAPBA gel by 0.5 M fructose, a strong competitor for diol binding to PBA [29,30]. The absorbed amount of ARS was dependent on the incubation time of the gel in the dye solution, as illustrated by the inset in Fig. 7. The gel was saturated with the dye after 4 h at room temperature (22 °C), using 4.5 mM ARS. The concentration was chosen to be higher than the equilibrium dissociation constant of PBA–ARS complex (6.7×10^{-4} M at pH 7.0 [18]) and, therefore, was supposed to ensure the complete saturation of the PBA ligands of the reactive gel.

Fig. 7 illustrates the desorption kinetics of ARS absorbed in the AAm–NAAPBA gel from 0.1 M sodium phosphate buffer pH 7.0. Ethyl Orange (EO), a dye without diol group (see Fig. 8), used as a control absorbate was unable to form specific complexes with PBA ligands. Absorption of ARS within the gel was much higher compared to EO, though both the dyes were water-soluble aromatic compounds of comparable molecular weight (342 and 333 g/mol, respectively). One may anticipate a combination of two reasons for the higher absorbed amount of ARS: first, a specific interaction of ARS with the immobilized PBA ligands and, second, a tendency to self-association displayed by ARS in aqueous solution [31]. The self-association of ARS resulted in its higher non-specifically adsorbed amount and lower desorption rate during the washing of the stained gel with the phosphate buffer solution, compared with EO (see Fig. 7). The specifically absorbed ARS could be effectively eluted from the gel in the presence of 0.5 M fructose. Obviously, no specific absorption of EO in the gel was found (see Fig. 7). The amount of specifically

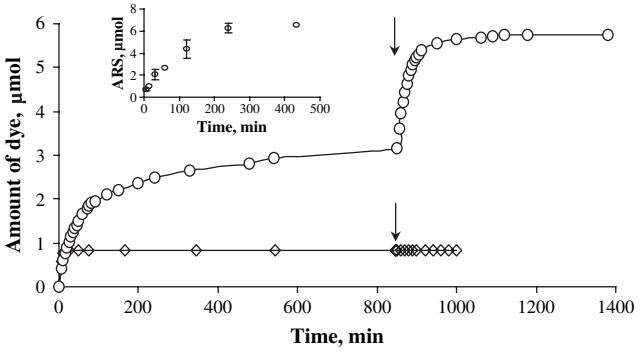

Fig. 7. Desorption of ARS (○) and Ethyl Orange (◇) from AAm–NAAPBA gel into 0.1 M sodium phosphate buffer solution, pH 7.0. The arrows indicate immersion of the gel into the buffer solution containing 0.5 M fructose. The inset illustrates the total amount of ARS adsorbed by the AAm–NAAPBA gel as a function of time. The error bars indicate the standard deviation of three independent measurements. Thickness of the gels is 0.13 mm.

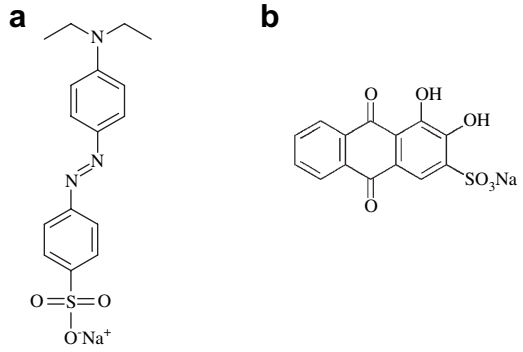

Fig. 8. Structural formulas of Ethyl Orange (a) and Alizarin Red S (b).

adsorbed ARS was found to be 160 ± 35 μmol/mL in the gel and agreed well with the amount of immobilized PBA 160 ± 10 μmol/mL gel estimated as described in Section 2.4. This indicated a 1:1 stoichiometry of the reaction at pH 7.0, which agrees well with the value of equilibrium constant for dissociation of the complex between ARS and PBA at pH 7.0 [18]. The 1:1 stoichiometry suggests a good accessibility of PBA ligands in the AAm–NAAPBA gels and their high reactivity with diols. These properties can originate in the highly porous structure and good permeability of the gels for water-soluble organic compounds.

3.3. Kinetics of the gel optical response to glucose

The rate of optical response exhibited by AAm–NAAPBA gel in the presence of sugars (see Figs. 4 and 5) can be a consequence of at least three different processes: (1) diffusion of a sugar into the gel, (2) affinity complex formation between the sugar and PBA groups, and (3) structural rearrangements of the gel due to the increasing concentration of negative charges resulting in gel hydration and swelling. In the case if the affinity complex formation or the structural rearrangements is the rate-limiting process, the overall rate of the OD changes of the gel would not depend on the gel thickness (l). To find out if the above processes determine the rate of the gel optical responses, they were investigated using the gels with different thicknesses. The fractional attainment of equilibrium (F) was studied as a function of time as described in Section 2.9. The slops of linear $F/\sqrt{t}$ dependences (see Eq. (3)) decreased with increasing l, indicating the diffusion controlled type of the reaction, at least for the studied thicknesses of the gels (see Fig. 9a).

Further, the apparent diffusion coefficients calculated according to Eq. (3) were found to be independent of the gel thickness for the groups of gels 1, 2 and 3 (see Table 1) and equal to $2.3 \pm 0.9 \times 10^{-7}$ cm^2/s. At the same time, the apparent diffusion coefficients calculated for thin gels of group 4 (see Table 1) were somewhat lower ($5.3 \pm 0.3 \times 10^{-8}$ cm^2/s) than those of groups 1, 2 and 3. This might reflect an input from the affinity binding kinetics of glucose or the structural rearrangements noticeable in the thin gels. As the initial parts of kinetic curves could not be reliably registered for the fast reaction of glucose with the thin gels of group 4, the

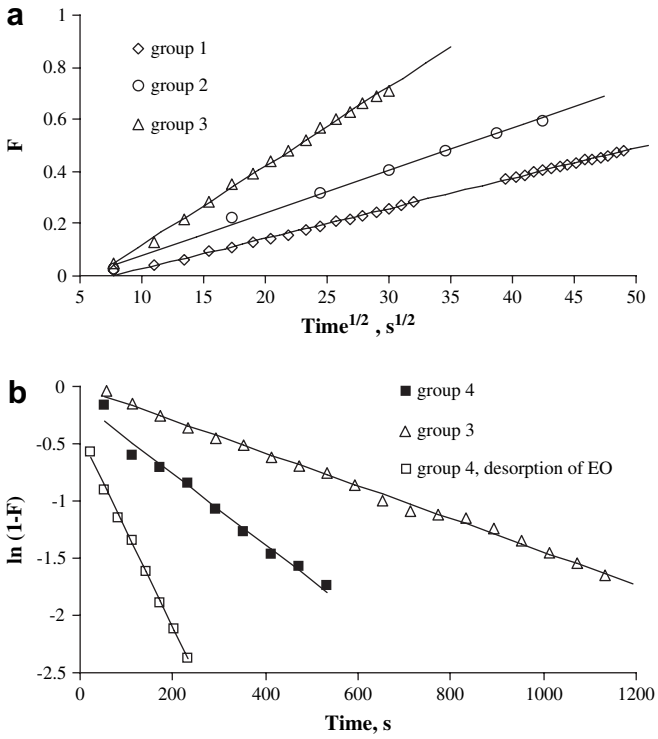

Fig. 9. (a) Plot of F versus $t^{1/2}$ for the glucose absorption by AAm–NAAPBA gels from 40 mM glucose solution in 50 mM sodium phosphate buffer solution, pH 7.3. (b) Plot of $\ln(1-F)$ versus t for the glucose absorption by AAm–NAAPBA gels from 40 mM glucose solution in 50 mM sodium phosphate buffer solution, pH 7.3 and EO desorption from the gel in 50 mM sodium phosphate buffer solution, pH 7.3. Thicknesses of the gels: 1.0 (group 1), 0.83 (group 2), 0.35 (group 3) and 0.13 (group 4) mm.

calculation of apparent diffusion coefficients were performed according to Eq. (5), see Section 2.9 and Fig. 9b. As follows from Fig. 9b, the rate of gel response to glucose is still faster in the gels of group 4 compared to the gels of group 3, as follows from the slopes of corresponding $\ln(1-F)$ versus t dependences.

To detect a possible input of the structural rearrangements of the gel into the overall rate of the gel swelling and shrinking, the following experiment was carried out. An AAm–NAAPBA gel of group 3 was equilibrated with 50 mM glucose in 50 mM sodium phosphate buffer, pH 7.5, and transferred into a spectrophotometric cuvette filled with the sugar-free buffer. The release of glucose and the optical density changes were registered simultaneously, the glucose assay being performed as described in Section 2.8. The increase in glucose concentration and optical density of the gel were expressed as dimensionless values of the fractional attainment of equilibrium (F) and are illustrated in Fig. 10. The experiment demonstrated that the changes in the gel optical density fell behind the glucose depletion: the diffusion of glucose out of the gel was faster than its optical response. This means that the process of structural rearrangements of the gel caused by its hydration or dehydration can contribute to the overall rate of the gel response. The input of the structural rearrangements might become noticeable in the thin gels of group 4 as reflected by the lower values of the apparent diffusion

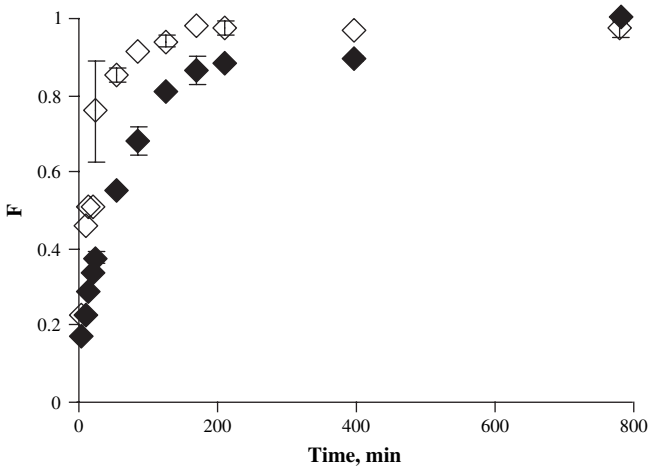

Fig. 10. Release of glucose from the AAm–NAAPBA gel in 50 mM sodium phosphate buffer solution, pH 7.5: (◆) the gel optical density change normalized to the maximum value; (◇) concentration of the glucose in the contacting buffer solution normalized to the maximum value. The error bars indicate the standard deviation of three independent measurements. Thickness of the gels is 0.35 mm.

coefficient, see Table 1. It seems realistic that in very thin boronate-containing gels like 5 μm-thick gels described in literature [2], the rate of affinity binding or the rate of the structural rearrangements can determine the overall rate of the gel response.

The exact estimation of the glucose pore diffusion coefficients into the AAm–NAAPBA gels is difficult because the glucose absorption and the gel swelling take place simultaneously, so that the calculated diffusion coefficient cannot be ascribed to any definite state of the gel porosity and hydration. The diffusion coefficients listed in Table 1 are, therefore, approximate values, that need to be confirmed by independent experiments. This can be done using a non-interactive organic absorbate, such as EO. Interestingly, the diffusion coefficients of glucose (6.7×10^{-6} cm^2/s [32]) and ionic dyes such as EO or methyl orange (6.9×10^{-6} cm^2/s [33]) in pure water are almost the same. The pore diffusion of EO would, therefore, resemble that of non-interacting sugars. Since EO can be easily desorbed from the gel (see Fig. 7), the pore diffusion coefficient was estimated from the profile of EO desorption from a gel of group 4, using Eq. (5) (see Fig. 9b). The pore diffusion coefficient calculated for EO ($D_p = 1.4\times10^{-7}$ cm^2/s) was similar and even somewhat lower than the apparent diffusion coefficients of glucose into the gels of groups 1–3 given in Table 1. This confirms that diffusion of glucose is likely the rate-limiting process in the response displayed by the relatively thick gels (0.35 mm and thicker) and the apparent diffusion coefficients of glucose into the gels listed in Table 1 are acceptable characteristics of the diffusion processes. They can be used to compare the permeability and reactivity of the AAm–NAAPBA gels with some other gels or polymer films containing PBA. For example, the effective diffusion coefficient of fructose into poly(aniline boronic acid) film obtained by Shoji et al. was 2×10^{-12} cm^2/s [22], that is about 5 orders of magnitude lower than those estimated in the present study,

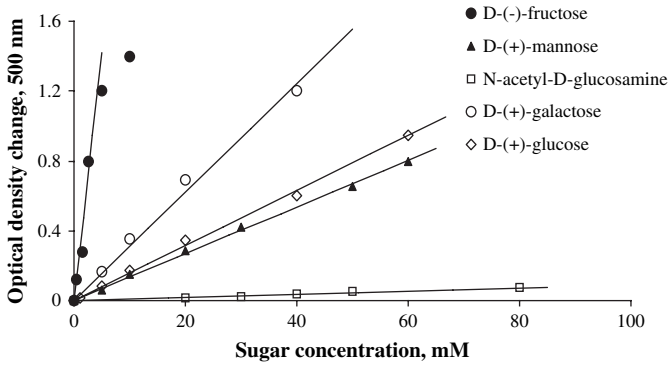

Fig. 11. Optical responses of AAm–NAAPBA gel to the different concentrations of the sugars in 50 mM sodium phosphate buffer solution, pH 7.3. Thickness of the gel is 0.35 mm.

probably due to a low porosity of the polyaniline films. Comparison of our results with the diffusion coefficient of glucose into the agarose films (6.5×10^{-6} cm^2/s), calculated by Zhang et al. [34], can be an evidence for comparable permeabilities of AAm–NAAPBA gels and the gels of agarose known to be highly porous and permeable.

3.4. Sensitivity of AAm–NAAPBA gels to different sugars

It is known that PBA binds to a wide variety of diols. Configuration of diol groups in the sugars strongly effects the binding affinity [29,30]. We have tested sensitivity of AAm–NAAPBA gels to several different sugars. As follows from Fig. 11, the sensitivity of the gels decreased in the series: D-fructose > D-galactose > D-glucose > D-mannose > *N*-acetyl-D-glucosamine. With the exception of the effects provided by glucose and mannose, this series is in good agreement with known equilibrium association constants of the sugars with phenylboronate ion: 4370 M^{-1} (fructose), 276 M^{-1} (galactose), 110 M^{-1} (glucose) and 172 M^{-1} (mannose) [28]. Qualitatively, our results are similar to those reported earlier by Asher et al. [16] and Lee et al. [13]. Equilibrium association constants of *N*-acetyl-D-glucosamine to PBA could not been found in the literature. In the molecule of *N*-acetyl-D-glucosamine, there exist the only option for phenylboronate binding to the 4,6-diol group characterized by very low local association constant of 3 M^{-1} [30].

The optical responses of AAm–NAAPBA gels were linearly dependent on sugar concentration from 1 to 40–60 mM for galactose, glucose, mannose and *N*-acetyl-D-glucosamine. Such proportionality is the advantage of optical detection of sugars over the methods based on the measurements of the gel swelling. As mentioned above, the reported volume changes of the gels were proportional to sugar concentration in a relatively narrow concentration range from 1 to 12 mM [13,16].

4. Conclusions

We have found that thin semitransparent polyacrylamide gels containing immobilized NAAPBA display optical response to sugars present in the contacting aqueous solution. The optical response of AAm–NAAPBA gels to fructose, galactose, glucose, mannose and *N*-acetyl-D-glucosamine was directly proportional to sugar concentration from 1 to 40–60 mM, whereas the sensitivity of gels to sugars decreased in the above series of compounds. Linear optical response of the AAm–NAAPBA sensors to glucose has been attained, therefore, in a wider range of sugar concentrations, compared to the responses of the boronic acid-based holographic or crystalline colloidal array sensors. The introduction of a bifunctional cross-linker to the polymer mixture affected the sensitivity of optical response, apparently due to increasing elastic resistance of the gel network to swelling. The concentration of immobilized PBA ligand in the gel optimal for the strong and quick optical response was found to be ca. 160 μmol/mL gel. Pore diffusion coefficient of a non-interactive solute, Ethyl Orange, indicated a good permeability of the gel for small water-soluble organic molecules, comparable with permeability of macroporous agarose gels. Diffusion of glucose into the gel determined the rate of optical response of the thicker gels (0.35–1 mm), whereas affinity complex formation between the sugar and PBA groups, or structural rearrangements of the gel due to its enhanced hydration in the presence of binding sugar seemed to input the rate of optical response observed with the thin gels (0.1 mm). The studied AAm–NAAPBA gels exhibited good stability during a long time of use and allowed to perform many tens of sugar assays with a single gel. The linear response and stability of the sensor gels make them applicable to various glucose sensing techniques such as measurement of glucose in human blood plasma [15] or other physiological fluids. Owing to the ability of NAAPBA-containing copolymers to interact specifically with polysaccharides [24], the gels can be also considered as potential deliverers of carbohydrate drugs.

Acknowledgements

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