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Protein–boronic acid conjugates and their binding to low-molecular-mass *cis*-diols and glycated hemoglobin

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Abstract

Different methods for covalent linkage of phenylboronic acid (PBA) to structural proteins and enzymes are presented. Protein–PBA conjugates, free in solution or immobilised on magnetizable polymer particles, were tested for their binding of D-sorbitol, D-mannose and glycohemoglobin (GHb). Similarly, alkaline phosphatase–PBA conjugates were used in an attempted enzyme-linked sorbent assay for the detection of GHb. Affinity chromatography on immobilised D-mannose and gel chromatographic studies of protein–PBA complexes with [^{14}C]sorbitol, clearly illustrated a low affinity of the interaction studied. Glycated hemoglobin could not be detected using the enzyme-linked sorbent assay approach. However, GHb was found to be specifically retained on columns filled with protein–PBA-coated particles as affinity matrix, enabling the glycation level of blood samples to be determined.

1. Introduction

Affinity chromatography using immobilised boronic acid has found its use in various fields of applications dealing with the problem of separating biomolecules [1–6]. Similarly, boric acid–diol interaction in solution has been employed in electrophoresis and ion-exchange chromatography [7–10].

The principle for their use is their well-known specificity for the 1,2-*cis*-diol configuration found in various biomolecules. The presence of this configuration in simple carbohydrates, polysaccharides and glycoproteins as well as in vitamins, catechols, nucleosides, nucleotides and nucleic acids, renders it possible to separate and purify these compounds [1–11]. Although boronic acid affinity chromatography has proved efficient in separating several of these compounds, both the nature of the solid matrix, the boronic acid derivative used as well as the coupling chemistry applied have proved to be of importance for their successful use [1,12,13].

A significant application of "boronic acid–*cis*-diol" interaction is found in the separation of glycohemoglobin from other non-glycated hemoglobin variants [6]. Glycohemoglobin is a well-known analyte in clinical chemistry and has wide acceptance as a long-time index of diabetic control. The affinity method using immobilised phenylboronic acid residues does not suffer from interferences displayed by several of the alter-

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native methods. Advantages are first of all related to low sensitivity towards variation in analytical parameters such as pH and temperature. The method is also insensitive towards the presence of interfering Hb species like the labile pre-glycated aldimine hemoglobin variant (HbA_{1d}), HbF and various abnormal Hbs [14,15]. However, based upon chromatography on minicolumns, the assay throughput is low and the method is not easily automated without the requirement of advanced and expensive equipment.

As hydrophilic components proteins, modified to contain boronic acid groups, bear the potential of reacting with glycated molecules in solution, making it possible to develop and utilise new principles for the determination of *cis*-diol analytes. By coating the microtiter-plates with hemoglobin-binding molecules, such as anti-hemoglobin antibodies and haptoglobin, we made use of alkaline phosphatase–phenylboronic acid conjugates in an attempt to develop an enzyme-linked sorbent assay for glycohemoglobin. Correspondingly, in order to find new hydrophilic boronic acid affinity supports, the results of utilising immobilised protein–phenyl boronic acid conjugates (protein–PBA) on magnetizable polymer particles are presented.

2. Experimental

1 - Ethyl - 3 - (3 - dimethylaminopropyl)carbodiimide hydrochloride (EDC), bis(sulfosuccinimidyl)suberate (BS^3) and Azomethine H were purchased from Pierce Europe (Oud-Beijerland, Netherlands). 1,1'-Carbonyldiimidazole (CDI) and glutardialdehyde (GDA) were from Fluka (Buchs, Switzerland). Bovine alkaline phosphatase, nitrophenyl phosphate (NPP), casein, humane haptoglobin and 3-aminophenyl boronic acid-hemisulfate (APBA) were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Boehringer (Mannheim, Germany) and chicken anti-hemoglobin-antibodies (anti-Hb-Ab) from Immunsystem (Uppsala, Sweden). To minimise any interaction with PBA due to the presence of glycated proteins both BSA, alkaline phosphatase, anti-Hb-Ab and haptoglobin were eluted through boronic acid columns before use.

Amine-functionalized magnetizable polymer particles, 0.5–1.5 mm in size (BioMag M4100), were obtained from Advanced Magnetics (Cambridge, MA, USA), and hydroxyl-functionalized particles (Dynospheres XP-6006, nominal size: 2.8 mm ± 10%) from Dyno Particles (Lillestrøm, Norway).

Human hemoglobin (Hb) samples with glycohemoglobin levels ranging from 5 to 20% (determined by boronate affinity columns, Pierce Europe) were purified from freshly collected blood samples using EDTA as anticoagulant. Erythrocytes were washed five times with ten volumes of saline water, then hemolyzed adding Triton X-100 to a final concentration of 0.05% (v/v). Hemolysates were purified by gel chromatography on Sephacryl HR 200 (Pharmacia Biotech Norden, Sollentuna, Sweden) using 0.05 *M* sodium-phosphate buffer, 0.10 *M* NaCl, pH 7.4 as eluent. Purified Hb was stored frozen until used. Additionally, "non-glycated" hemoglobin samples (GHb level $< 0.2\%$) as well as samples of highly purified glycohemoglobin were used (evaluated by ion-exchange chromatography and signal integration to be of $> 90\%$ chromatographic purity regarding the HbA_{1c} component, i.e. glycation of Hb at the N-terminal valine of the β-chain [16]) —publication in preparation. Water used in all solutions was of Milli-Q quality, all other chemicals were of analytical grade.

2.1. Preparation of protein–PBA conjugates

The following procedures are representative for the preparation of protein–PBA conjugates used in this study. After preparation the protein–PBA conjugates were first dialysed against 0.02 *M* sodium-phosphate buffer, 0.15 *M* NaCl, pH 7.4, then finally against the buffer used for binding studies or particle immobilisation.

Before conjugation, casein was dissolved to a concentration of 3 mg/ml in 0.2 *M* sodium carbonate–bicarbonate buffer, pH 8.5 (facilitated by heating to 45°C). The resulting protein solution was filtered (0.2 μm), then dialysed

excessively against 0.15 *M* NaCl and purified water before being lyophilised. This treatment made it possible to achieve solutions with a casein concentration of 20 mg/ml.

Bis(sulfosuccinimidyl)suberate (BS^3)

A 10-mg amount of protein in 1 ml 0.4 *M* sodium-bicarbonate buffer, pH 7.5 was added to 100 μl of a 10–60 m*M* APBA solution and cooled on ice. Under constant stirring 1–3 mg BS^3 was added and the solution left to react overnight at room temperature before being dialysed.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)

A 0.5–2 mg sample of EDC was added under constant stirring to 1 ml ice-cold 0.05 *M* 2-[N-morpholino]ethanesulfonic acid, pH 6.0 containing 10 mg protein and 1–10 mmol APBA. After 2 h the pH was adjusted to 7 and the resulting solution left to react overnight at room temperature before being finally dialysed.

Glutaraldehyde (GDA)

A 300-μl aliquot of a freshly prepared solution of glutardialdehyde (0.050–0.125 *M*) in 0.01 *M* pyridine buffer, pH 7.0 was slowly added to a mixture of 1.5 ml APBA (10–25 m*M*) and 1.5 ml casein (20 mg/ml), both in pyridine buffer. The GDA/APBA ratio in these conjugations was kept constant at 1:1. The solution was gently agitated for 4 h, then left overnight before being dialysed. This procedure was optimised to be used with casein. Using other proteins excess GDA may have to be adjusted to avoid precipitation due to cross-reactivity and protein polymerisation.

Diazonium ion coupling

Diazotisation of APBA and conjugation to proteins was prepared following the procedure described by Tijssen [17], using 5–25 m*M* APBA solutions and protein (5 mg/ml) in 0.5 *M* sodium bicarbonate–carbonate buffer, pH 8.0.

2.2. Particle immobilisation of protein–PBA conjugates

Immobilisation of protein–PBA conjugates on BioMag M4100-polymer particles was performed using glutardialdehyde in 0.01 *M* pyridine, pH 6.0, following a modified version of the procedure reported by Weston and Avrameas [18].

Immobilisation on Dynospheres XP-6006 particles was performed using 1,1′-carbonyldiimidazole (CDI)-activated particles prepared as described by McConway and Chapman [19].

Immobilisation of both ^{125}I-labelled and non-labelled BSA and casein was used to estimate total protein-binding capacity of the activated matrices. Radiolabelled protein, prepared with the Iodo-gen method [20], was also used to control immobilisation, test unspecific binding and follow the stability of the protein-coated particles.

2.3. Boron analysis

The boron content of the protein–PBA conjugates as well as protein–PBA-coated particles was determined following the method presented by Gaines and Mitchell using azomethine H [21].

2.4. Binding studies: protein–PBA–*cis*-diols

The different protein–PBA conjugates were tested for their diol-binding functionality in different experimental set-ups:

(1) Casein/BSA-PBA: the binding of D-[^{14}C]sorbitol (Amersham Laboratories, Amersham, UK, specific activity: 10.1 GBq/mmol, 274 mCi/mmol, 99.3%) was studied by gel permeation chromatography on Bio-Gel P2, P60 (Bio-Rad, Richmond, CA, USA) and Sephacryl HR 200. Eluent 0.25 *M* ammonium acetate buffer, pH 8–9.

(2) BSA/casein–PBA: the binding of highly purified HbA_{1c} (>90%) was studied by gel permeation chromatography.

(3) Casein–PBA: by affinity chromatography on immobilised D-mannose (on agarose, Pierce

Europe); buffer systems ranging from pH 7.5 to 9 were applied.

(4) Alkaline phosphatase–PBA: tested in an enzyme-linked sorbent assay using microtiter wells coated with either chicken anti-Hb antibodies haptoglobin or hemoglobin (HbA_{1c} level 5–90%); alkaline phosphatase–PBS conjugates with PBA/enzyme ratios of 5–15:1 were used in this study. A solution of 0.1 *M* sodium phosphate, 0.15 *M* NaCl pH 8–9, or 0.1 *M* ammonium acetate, pH 8–9 were used as buffers and NPP in 0.1 *M* glycine, 1 m*M* $MgCl_2$, 1 m*M* $ZnCl_2$, pH 9.8 as enzyme substrate. The binding of enzyme–PBA conjugate to GHb was studied either directly on immobilised HbA_{1c}, or after mixing with hemoglobin followed by subsequent immobilisation of the resulting alkaline phosphatase–PBA–GHb complex by haptoglobin or chicken anti-Hb-Ab.

(5) BSA/casein–PBA after particle immobilisation: tested for their binding of glycated hemoglobin, either using photometric reading of particle-treated hemoglobin solutions or small chromatographic columns (1.0 × 1.0–4.0 cm) containing protein–PBA-coated particles as affinity matrix. Particle treatment of Hb-solutions was performed directly in cuvettes by adding (1–15 mg/ml) protein–PBA-coated particles. After incubation, particles were separated at one side of the cuvette allowing photometric reading to quantify bound glyco-hemoglobin. Absorbance readings at 545 and 575 nm were performed both adding particles to the Hb-solution and immediately after particle separation. Binding of GHb was monitored as reduced hemoglobin absorptivity after particle separation.

Using protein–PBA-coated particles as column affinity matrix, binding capacity for glycated hemoglobin and determination of association constant for the studied interaction were performed. For the quantitative determination frontal analysis [22] and competitive zonal elution volumes according to Chaiken [23] were used. Highly purified HbA_{1c} and non-glycated Hb were used in these experiments. Ligand (PBA) concentration of the affinity support was based upon analysis of elemental boron.

2.5. Electrophoretic studies

Polyacrylamide gel electrophoresis (PAGE) studies of the bound hemoglobin fraction were performed using a PhastSystem (Pharmacia Biotech Norden). Native PAGE was carried out using pre-cast PhastGel Native PAGE 8-25 or 10-15. Isoelectric focusing was performed using PhastGel IEF 5-8. The bound glycohemoglobin fraction eluted from commercial boronate affinity columns was used as reference.

3. Results

3.1. Protein–PBA conjugation

The conjugation methods applied were all able to incorporate low to medium (5–20 molecules/BSA) amounts of 3-aminophenyl boronic acid into the proteins used, although generally the GDA method was the most efficient one. Fig. 1 illustrates the incorporation of PBA into casein by increasing amounts of GDA–APBA used. To achieve the solubility of casein necessary for the conjugation procedures presented, dialysation with subsequent lyophilisation was found to be essential. At most these experiments resulted in a PBA/BSA ratio of approximately 50:1 and a PBA/casein ratio of 22:1. Under comparable reaction conditions the observed incorporation of PBA into proteins depended upon the labelling method used. As high incorporation of PBA was a main goal, the GDA conjugation method was preferred, especially in combination with casein.

For comparison, the highest PBA/BSA ratios obtained using the EDC and the BS^3 methods was 23:1 (coefficient of variation, C.V. ca. 15%) and 21:1 (C.V. ca. 17%), respectively. Although PBA/BSA ratios as high as 20:1 also were achieved using the diazonium ion conjugation method, this method was clearly the least reproducible of the methods applied (C.V. ca. 30%, under "controlled" conditions). The reluctance to the diazonium ion conjugation method was mainly related to the formation of red-

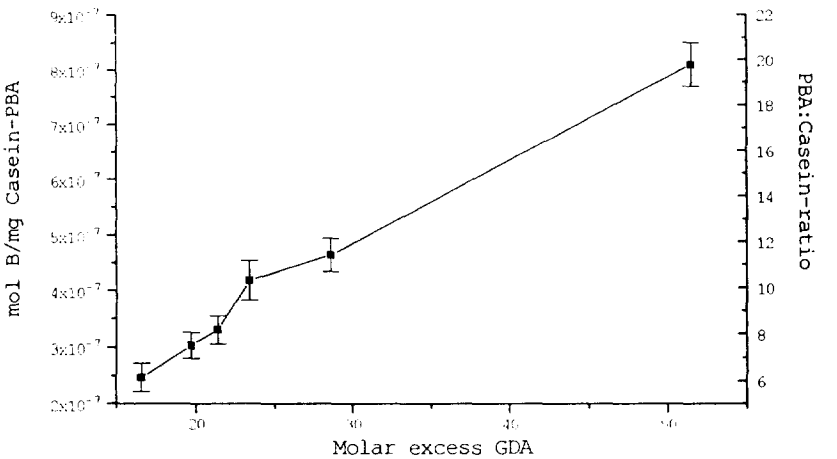

Fig. 1. PBA–casein conjugation. Incorporation of PBA as a function of molar excess GDA (relative to amount of protein) used under conjugation. GDA/APBA = 1:1. The incorporation of boron in the modified proteins, the PBA/protein ratio, is determined from the boron content found in the different protein–PBA conjugates (given as mol B per mg casein–PBA), using 25 kDa as the molecular mass of casein.

coloured azo-dyes of APBA, consuming considerable amounts of phenylboronic acid unless the pH was critically controlled. A simple ranking of the conjugation methods and their applicability based upon incorporation of APBA and low by-product formation, follow in decreasing order: GDA > EDC, BS^3 > diazonium ion coupling.

Although the necessity of reducing the said unstable Schiff base formed during GDA-conjugation may be questionable [24,25], some of the protein–PBA conjugates prepared by the GDA method were alternatively treated with $NaBH_3CN$ and $NaBH_4$. However, analysis and behaviour of these conjugates were all indistinguishable from those of untreated material.

3.2. Particle immobilisation

Although both tolyl chloride and tosyl chloride were employed in preliminary experiments as activation reagents for the XP-6006 particles (for immobilisation of APBA and different non-protein–PBA proteins, results not shown), the CDI method presented was found to be the most appropriate one for these particles. This method resulted in the highest immobilisation yield: however, as illustrated from the results summarised in Table 1, M3100-particles were found to be superior with respect to both immobilisation yield and incorporation of boron. Highly reproducible immobilisation of PBA–protein was achieved using this matrix (C.V. of the immobilisation yield $< 10\%$).

3.3. Binding studies

Due to the rather low affinity of boronic acid–*cis*-diol interactions [26,27], several protocols were used to test and verify the boronic acid functionality of the prepared BSA/casein–PBA conjugates. Results obtained using non-immobilised protein–PBA conjugates are depicted in Figs. 2 and 3. *cis*-Diol affinity chromatography of a BSA–PBA conjugate (PBA/protein ratio 30:1) on immobilised D-mannose is illustrated in Fig. 2. No addition of competing *cis*-diol containing molecules was necessary to displace the conjugate from the column, leaving low affinity or reduced accessibility of the boronic acid-D-mannose-residues as reasonable interpretations of the results. Fig. 3 is illustrating protein–PBA–sorbitol interaction, showing the alteration of the chromatographic (gel permeation) elution profile

Table 1
Particle immobilisation of protein–PBA conjugates—summarised results

Particle	Maximum immobilisation (μg protein–PBA/mg part.)	Reproducibility[a] (C.V., %)	Boron content (mol B per mg part.)
XP 6006 (2.8 mm)	55 μg (BSA–PBA)	< 15%	$< 2.5 \cdot 10^{-8}$
M2100 (0.5–1.5 mm)	100–180 μg (BSA–PBA) 100–250 μg (casein–PBA)	< 10%	$2–18 \cdot 10^{-8}$

[a] Immobilisation yield, coefficient of variation (%).

of [^{14}C]sorbitol by adding casein–PBA conjugate to the sample.

No separation of protein–PBA–HbA$_{1c}$ complexes was accomplished using gel-permeation, nor was it possible to detect significant binding of glycohemoglobin in the enzyme-linked sorbent assay approach using the prepared alkaline phosphatase–PBA conjugates. Similarly, unless large amounts of particles were used (> 10 mg/ml), negative results regarding binding of GHb were also experienced using the particle-immobilised protein–PBA conjugates in solution. However, using protein–PBA-coated particles as affinity matrix in chromatographic columns, glycohemoglobin binding was clearly detected. This is exhibited in Fig. 4, showing the elution of retarded glycohemoglobin as the buffer is changed to one containing sorbitol. Although encumbered with some uncertainty, the binding capacity (± S.D.) of the affinity matrix, determined by frontal analysis, using 0.25 *M* ammonium acetate buffer, pH 9.0 and a linear flow-rate of 0.2 cm/min was estimated to be as low as 0.15 ± 0.026 μg of GHb per mg particles. Correspondingly, an overall apparent association constant of 2800 ± 440 M^{-1} at pH 9.0 and 2200 ± 330 M^{-1} at pH 8.0 was found, illustrating

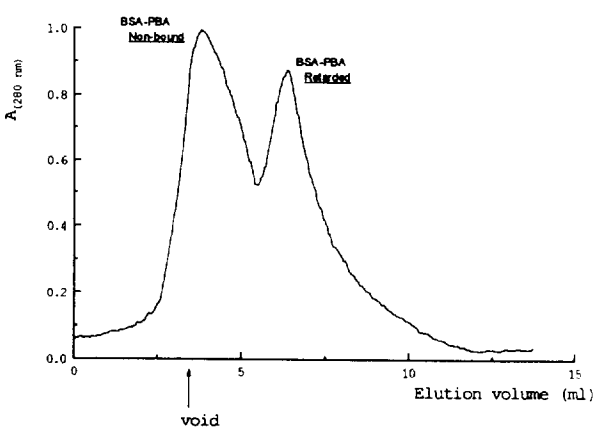

Fig. 2. Affinity chromatography of BSA–PBA conjugate on immobilised D-mannose. BSA–PBA conjugate with PBA/protein ratio 30:1 (500 μl, 1.5 mg/ml). Column: 5 × 1 cm I.D., buffer 0.25 *M* ammonium acetate, pH 8.0, flow-rate 0.3 ml/min. Bound and retarded fractions of the protein–PBA sample are indicated.

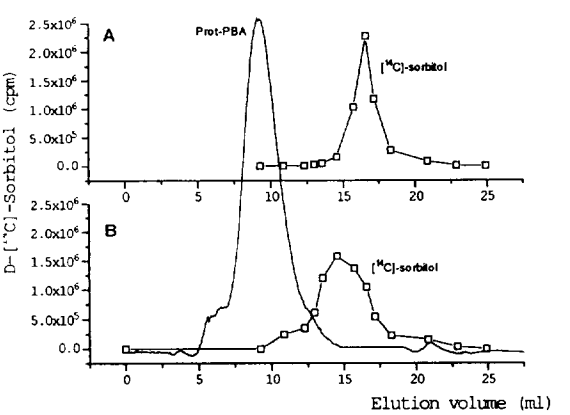

Fig. 3. Gel permeation study of D-[^{14}C]sorbitol binding to casein–PBA on Sephacryl HR 200. (A) D-[^{14}C]sorbitol alone (5 mCi). (B) D-[^{14}C]sorbitol/casein–PBA mixture (D-[^{14}C]-sorbitol, 5 mCi, and 1.7 mg casein–PBA conjugate, PBA/protein ratio 15:1, in 500 μl, incubated for 15 min before being separated). Column 30 × 1 cm I.D., buffer 0.25 *M* ammonium acetate, 50 m*M* $MgCl_2$, pH 8.5, flow-rate 0.5 ml/min. No alteration of the elution profile of [^{14}C]sorbitol was seen using native non-modified proteins or when additional non-labelled sorbitol was added. — = *A* (280 nnm); □ = counts [^{14}C]sorbitol.

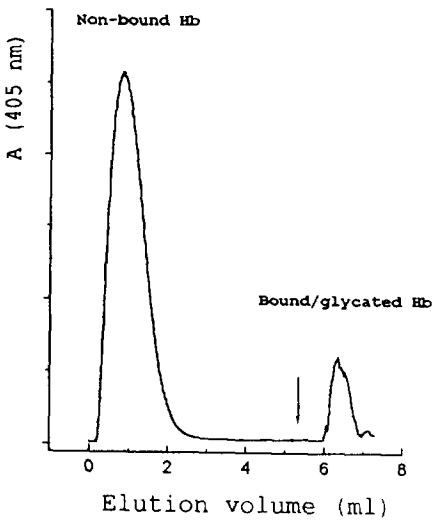

Fig. 4. Separation of human whole blood hemolysate on protein–PBA affinity column. Hemoglobin sample (0.1 mg Hb, 15% GHb as determined by affinity chromatography, Pierce Europe) separated on column (2 × 1 cm I.D.) filled with casein–PBA-coated particles (PBA/protein ratio 18:1, $15 \cdot 10^{-8}$ mol B/mg part.). Buffer 0.25 *M* ammonium acetate, 50 m*M* $MgCl_2$, pH 8.5 (I), flow-rate 0.15 ml/min. Arrow indicates buffer change to GHb-elution buffer: 0.05 *M* Tris, 0.1 *M* D-sorbitol, pH 8.0. Bound (GHb) and unbound fractions of hemoglobin are indicated. The sample was prepared by diluting whole blood (collected using EDTA as anticoagulant) 1:25 in 0.25 *M* ammonium acetate, 50 m*M* $MgCl_2$, 0.1% Triton X-100, pH 8.5. A 20-μl aliquot of this solution was injected on the column. The unbound fraction was eluted using 5.3 ml ammonium acetate buffer (I), followed by 2 ml of GHb elution buffer to elute the bound GHb fraction. Estimated glycation level (by signal integration): 14.5% GHb.

a low PBA-ligand utilisation of the protein–PBA-coated particles.

4. Discussion

The choice of conjugation chemistry applied for the preparation of the presented boronic acid–protein conjugates strongly depends upon the goals to achieve. Very high incorporation of PBA into protein structures will naturally affect physical properties as solubility and hydrophobicity, and polymerisation occurs using homobifunctional reagents like GDA and BS[3].

Generally APBA behaves like aniline with a low acid equilibrium constant of its conjugate acid (pK_a approximately 4.7, evaluated by titration), and low conjugation pH can be used without protonation of the amine. For the BSA/casein–PBA conjugates high incorporation of PBA was the main goal. Cross-reactivity and polymerisation of the protein was of little concern as long as the solubility of the conjugate was acceptable. Since incorporation of PBA was easily controlled by varying the amount of GDA and APBA (Fig. 1), these conjugates were found to be the most suitable ones. For the preparation of the alkaline phosphatase–PBA conjugate, polymerisation and incorporation of PBA bear the potential of affecting enzyme activity after conjugation. Still, PBA/enzyme ratios of 10–15:1 prepared by the EDC and BS[3] conjugation methods did not result in measurable alteration of the enzyme activity.

As shown in Table 1, particle immobilisation of protein–PBA conjugates varied as was expected from difference in particle size, surface functional group density and the conjugation chemistry applied. Amine-functionalized particles (size 1–1.5 mm) resulted in the most flexible matrix for immobilisation. Unless using particles with the most highly substituted and modified protein–PBA conjugates in combination with low pH, they all acted as hydrophilic surfaces with low tendency of protein adsorption. This was quite contrary to what was experienced when PBA was immobilised directly on the surface functional groups without first using the hydrophilic protein matrix as support.

The *cis*-diol binding characteristics of the prepared protein–PBA conjugates in solution are clearly illustrated in Figs. 2 and 3. Using affinity chromatography on immobilised D-mannose only weak "protein–PBA–D-mannose" interactions could be detected; most of the conjugate eluted with only minor retardation. Similarly, the results illustrating the interaction of the protein–PBA conjugate with D[^{14}C]sorbitol are only indicative as it was impossible to separate the complex as a single fraction. Only a small, although significant, displacement of the D-[^{14}C]-sorbitol peak was observed when separating the

protein–PBA–sorbitol mixture compared to separating D-[^{14}C]sorbitol alone. As a result of this low affinity, high concentration of the reactants involved is therefore a prerequisite to achieve binding and formation of detectable protein–PBA–*cis*-diol complexes.

In addition to the specific boronic acid–*cis*-diol interaction, binding of glycohemoglobin to protein–PBA matrices may involve additional secondary interactions. Hydrogen bonding and ionic and hydrophobic interactions may all be of importance in this respect by tending to influence the strength and character of the binding. However, the existing association constant seems to be too small to allow the washing steps necessary in the enzyme-linked sorbent assay approach. Similarly, lack of separation of different molecular species by gel filtration are in all probability a result of fast association–dissociation equilibria involving the interacting species. Contrary to the sorbent assay approach, washing was not performed in the "particle/cuvette" experiments. Still, the main restriction to the absorption measurements after magnetic separation of "particle–protein–PBA–GHb" complexes, was the particle- and hemoglobin-concentration necessary in these experiments. To detect differences due to the binding of GHb, an unacceptable amount of particles had to be used. This in turn resulted in low reproducibility due to unspecific binding, separation problems and increased tendency of heme/iron(II) proto-porphyrin IX oxidation.

Binding of GHb to protein–PBA conjugates was most clearly demonstrated using affinity chromatography on protein–PBA-coated particles (Fig. 4). As evaluated by isoelectric focusing the bound hemoglobin fraction eluted with sorbitol was found to be identical to GHb eluted from commercial boronic acid affinity columns. Quantification of this GHb fraction by signal integration correlated well with the glycation level of the hemoglobin samples used and resulted in GHb levels similar to what was achieved using boronic acid affinity columns (Pierce Europe). However, chromatographic behaviour and ease of evaluation varied a lot depending upon linear flow-rate, buffer used and sample concentration. Most of these problems relate to the matrix itself and are a consequence of the low total binding capacity. This easily results in overloading of the columns if the amount of sample is not critically controlled. Lack of porosity of the particle matrix and low accessibility of the available PBA-ligands present in the protein network seem to be critical in this respect [28]. Both low percentage ligand utilisation due to reduced availability and the low affinity nature of the boronic acid interaction against *cis*-diols, seem to be the main restriction to the use of the described protein–PBA conjugates in separating and assaying *cis*-diol-containing molecules.

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