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An efficient preparation of polyanionic affinity agent and its evaluation for the measurement of glycated hemoglobin

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Abstract—An efficient method was developed for the preparation of polyanionic affinity agent (**3**), a key component in the measurement of glycated hemoglobin (GHb). Glycated hemoglobin is an important clinical marker for diagnosis of patients with diabetes and useful to monitor the management of disease. The affinity agent (**3**) was prepared based on coupling reaction between poly(acrylic acid) (**1**) and 3-aminophenylboronic acid (**2**) in water. The critical features of this polymeric affinity agent (**3**), such as size, boronic acid incorporation ratio and concentration, on the measurement of glycated hemoglobin were evaluated. It was found that the agent (**3**) prepared using poly(acrylic acid) (**1**) with 225 kDa molecular weight gave optimal GHb measurement. The performance test results demonstrated that the boronic acid incorporation ratio and concentration of affinity agent (**3**) play a critical role in the assay and determines the precision of glycated hemoglobin measurement.

1. Introduction

Diabetes mellitus has become a major health problem and is recognized as one of the leading causes of death and disability in the world.[1] This chronic disease is associated with high risk for developing serious complications of organs such as the eyes, kidneys, heart, and blood vessels.[2] It is caused by a group of metabolic disorders related to carbohydrate metabolism in which the glucose is under utilized and results in increase of its concentration, a condition known as hyperglycemia.[3] The increased levels of blood glucose concentration and subsequent changes in the structure and function of several proteins, based on non-enzymatic glycation, is the pathogenic hallmark of this crippling disease, which is reaching epidemic proportions.[3] Hemoglobin (Hb) is one of many proteins that undergo non-enzymatic glycation, predominantly at the N-terminal valine residue and subsequently converts to glycated hemoglobin (GHb).[4] Since the red blood cells are freely permeable to glucose, the rate of formation of GHb from hemoglobin is directly proportional to the concentration of glucose in blood. Additionally, the formation of GHb from hemoglobin is irreversible and therefore, the concentration of GHb constitutes a reliable and integrated measure of the average blood glucose concentration over the life span of red blood cells, that is, ~3 months.[3,4]

Clinical utility of the measurement of glycated hemoglobin is widely recognized and commonly used in medical practice to diagnose and monitor the long-term glycemic status in patients with diabetes.[5] The GHb test is also used to assess the quality of diabetes care and evaluate or monitor the management of the disease. The American Diabetic Association (ADA) recommends GHb testing at least twice a year in patients with stable glycemic control and more frequently for patients whose therapy has changed or is not meeting the glycemic goals. Thus, GHb is used as an index of mean glycemia during the preceding 2–3 months, which also serves as a key predictor of the risk of developing serious complications associated with diabetes.[3,6] Therefore, it is critically important to measure GHb for diagnosis and monitoring of patients with diabetes using an assay, which is reliable and has demonstrated good long-term

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precision. A variety of methods have been developed over the years, for the measurement of glycated hemoglobin, such as, ion-exchange chromatography,[7a] colormetric analysis,[7b] affinity chromatography,[7c] affinity extraction,[7d] affinity precipitation,[7e] non-separation affinity assay,[7f] and by mass spectrometry.[7g] Additionally, significant efforts from different research groups are ongoing to develop a variety of new diagnostic tools for the benefit of diabetic patients.[2b]

Wilson et al.[8] reported the development of a fully automated assay for measurement of glycated hemoglobin (GHb) on Abbott IMx® analyzer.[9] The technology of this high-throughput assay (Fig. 1) is based on the ability of boronic acid to form cyclic esters upon interaction with 1,2-*cis*-diols,[10] and utilizes polyanionic affinity agent (**3**) as a key component. This polymeric agent (**3**) is prepared from poly(acrylic acid) (PACA, **1**) and 3-aminophenylboronic acid (APBA, **2**). The presence of both boronic and carboxylic acid groups in reagent (**3**) is critically important for the agent to effectively capture glycated hemoglobin and also to separate it on a cationic solid-phase matrix, which is made-up of merquat. After the ion capture, the matrix is washed with the buffer containing a fluorescent dye, 4-methylumbelliferone (MU), to remove hemoglobin (Hb). Simultaneously, the IMx-optical assembly measures fluorescence quench by GHb, which is present on the matrix. The matrix is then washed with sorbitol, and using a second aliquot of the patient sample, the fluorescence quenching due to total hemoglobin (GHb and Hb) is measured. Based on the fluorescence measurements from GHb and total Hb, the percentage of GHb is calculated and reported as the standardized $\%HbA_{1c}$ values.[8,11–13] In this paper, we describe an efficient method for preparation of polyanionic affinity agent (**3**) and its evaluation in the measurement of glycated hemoglobin. Determination of the effect of affinity agent's (**3**) size, boronic acid incorporation ratio, and concentration on the measurement of glycated hemoglobin, are presented.

2. Results and discussion

Polyanionic affinity agent (**3**), which is the key component of GHb assay, was prepared (Scheme 1) by conjugation of poly(acrylic acid) (PACA, **1**) and 3-aminophenylboronic acid (APBA, **2**) in MES buffer.[8,11] The previously reported method involved the purification of crude polymeric conjugate (**3**) by tedious diafiltration process using taurine buffer and the product was isolated as concentrated solution. The concentrated product was further diluted with taurine buffer to afford the affinity reagent solution (**3**). However, this reported procedure[8] is not practical and produced variable quality of polyanionic affinity agent (**3**) as observed from different batches. Therefore, a new method was envisioned, which involves coupling of PACA (**1**) and APBA (**2**) in water and purification of the crude conjugate by precipitation and isolation of affinity agent (**3**) as a powder. The powdered agent (**3**) can then be dissolved in taurine buffer in required concentration to produce the affinity agent (**3**) solution for application in GHb assay.

2.1. Preparation of polyanionic affinity agent

Accordingly, the mixture of PACA (**1**) with an average molecular weight of 225 kDa and 3-APBA hemisulfate

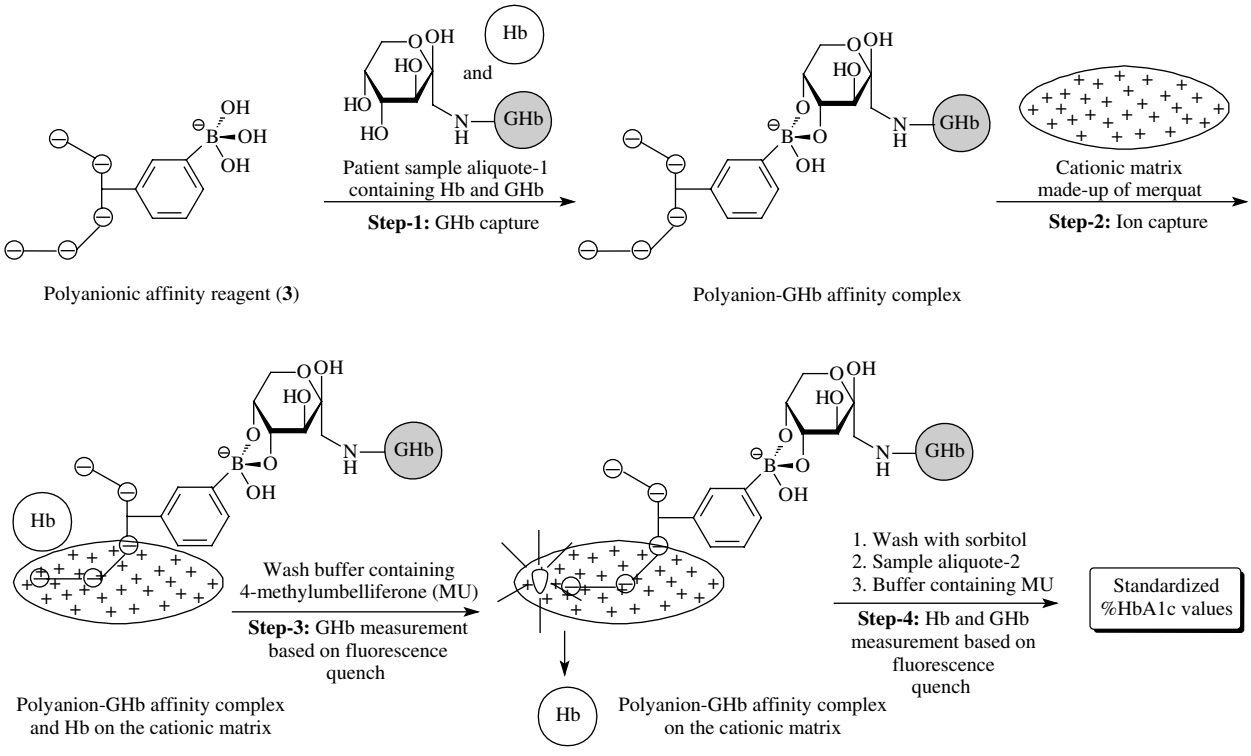
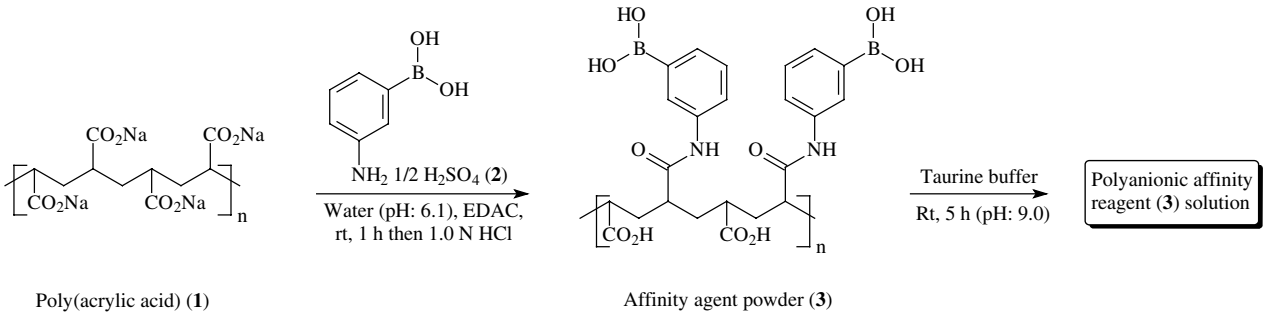


Figure 1. Measurement of glycated hemoglobin using polyanionic affinity agent (**3**).



Scheme 1. Preparation of polyanionic affinity agent (**3**).

(**2**) (Scheme 1) in water was treated with EDAC coupling reagent at pH 6.1. After stirring the reaction mixture for 1 h at room temperature, the mixture was acidified and the resulting precipitate was washed thoroughly with water. After drying the solid material, the affinity agent (**3**) was obtained in 95% yield as a colorless powder.[14] The powdered agent (**3**) was then dissolved in taurine buffer (pH 9.0) at room temperature for 5–6 h to afford the polyanionic affinity agent (**3**) solution. The concentration of reagent (**3**) solution was adjusted to 0.37 (range: 0.36–0.38, Table 1 and entry 3) based on the UV absorbance at 252 nm. The average percent boronic acid incorporation ratio (IR) in this polymeric agent (**3**) was found to be 37–39% based on the UV absorption and elemental analysis.[15]

The scope of this method was studied using different molecular weights of PACA (**1**) (Table 1) for the coupling reaction (Scheme 1). This study was also designed with a goal to evaluate the effect of the size of polyanionic affinity agent (**3**) on GHb measurement. Accordingly, the coupling reaction was carried out using different molecular weights of PACA (**1**), such as 30 kDa (entry 1), 100 kDa (entry 2), 250 kDa (entry 4), and 450 kDa (entry 5) by following the conditions described above. The crude conjugate was isolated by precipitation and the product (**3**) was isolated in 91–95% yield as a powder. The average percent incorporation ratio of boronic acid in each of these conjugates (entries: 1–2 and 4–5) was determined to be 37–39% based on UV absorbance and elemental analysis. The observed incorporation ratio (37–39%) is comparable to the affinity agent (**3**) prepared from 225 kDa PACA (entry 3) and consistent with the range of the molecular weight (30–450 kDa).[15] The powdered affinity agent (**3**), which was prepared from each of the different molecular weights of PACA (**1**) (entry: 1–5), was dissolved in taurine buffer to afford the affinity reagent (**3**) solution with concentration 0.36–0.38, based on the UV absorbance at 252 nm. Thus, the new method was found to be general and produced a consistent quality of affinity agent (**3**) using different molecular weights of PACA (**1**) in good yield. The procedure was efficient and eliminated the tedious diafiltration for purification,[8] and is amenable for large scale preparation of affinity agent (**3**).

2.2. Measurement of glycated hemoglobin

The polyanionic affinity reagent (**3**) solution prepared using different molecular weights of PACA (**1**) was tested (Table 1) for efficacy in glycated hemoglobin measurement using three controls on IMx® analyzer.[8,9] The three assay controls, which contain different levels of glycated hemoglobin, are low control with target value 8.3 and a range of 7.6–9.0, medium control with target value 12.6 and a range of 11.9–13.3, and the high control with target value 17.0 and a range of 16.3–17.7. The performance test was carried out in duplicate and the average $\%HbA_{1c}$ value was recorded for each control. It was found that polyanionic affinity agent (**3**) produced from different molecular weights of poly(acrylic acid) (**1**) read the three controls, differently. The reagents prepared using 30 kDa PACA (**1**, entry 1), and 450 kDa PACA (**1**, entry 5) gave performance results with all three controls out of their specified range. The reagents prepared from 100 kDa PACA (**1**, entry 2) and 250 kDa PACA

Table 1. Preparation of affinity agent (**3**) using different molecular weight PACA (**1**) and measurement of GHb

Entry	PACA (**1**) MW[a] (kDa)	Ratio of reagents PACA (**1**):APBA (**2**)[b,c] (equiv)	Affinity agent (**3**) UV (252 nm)	GHb measurement[d]		
				Low	Medium	High
1	30	1.0:0.485	0.37	7.4	11.7	16.1
2	100	1.0:0.485	0.37	7.9	12.2	16.3
3	225	1.0:0.485	0.37	8.1	12.6	17.0
4	240	1.0:0.485	0.38	7.9	12.0	16.3
5	450	1.0:0.485	0.38	6.7	11.5	16.0

[a] An average molecular weight.
[b] 3-Aminophenylboronic acid (**2**) was present as its hemisulfate salt.
[c] In all entries (1–5), 0.94 equiv of EDAC was used.
[d] GHb assay was performed using three controls, low (target: 8.3/range: 7.6–9.0); medium (target: 12.6/range: 11.9–13.3) and high (target: 17.0/range: 16.3–17.7).

(**1**, entry 4), although met the assay requirements, their performance results were at the very low end of the specified range. However, the polyanionic affinity agent (**3**) prepared from 225 kDa PACA (**1**, entry 4) produced the best results, which were in the range and close to the target values of each control (low, medium, and high), as desired. Therefore, the poly(acrylic acid) (**1**) with molecular weight of 225 kDa was selected for the measurement of GHb in subsequent studies.

The presence of both boronic and carboxylic acid groups are critically important for the affinity agent (**3**) to function effectively in GHb capture and measurement. To evaluate the effect of percent incorporation ratio of boronic acid on the measurement of GHb, we have prepared the affinity agent (**3**) using different ratios of 3-aminophenylboronic acid (APBA, **2**) (Table 2). The ratio of APBA (**2**) was ranging from 0.309 to 0.619 compared to poly(acrylic acid) (**1**, MW 225 kDa). Accordingly, the coupling reaction with different ratios (entries 1–6) was carried out under the optimized conditions using 1.94 equiv, of EDAC when compared to APBA (**2**). The powdered conjugates (**3**) prepared using different ratio of APBA (**2**) were then dissolved in taurine buffer and the polyanionic affinity reagent (**3**) solution was prepared with a concentration of 0.36–0.38 based on UV absorbance. It was found that the affinity agent (**3**) prepared using 0.309 equiv of APBA (**2**) had 24–27% boronic acid incorporation ratio (entry 1) based on UV absorbance and elemental analysis. The incorporation ratio (IR) for the agent (**3**) prepared using 0.619 equiv of APBA (**2**) (entry 6) was 42–49%. As expected, the agent (**3**) prepared using higher than 0.309 equiv and lower than 0.619 equiv of APBA (**2**) had boronic acid incorporation between 27% and 49% (entries: 2–5). The gradual increase in the percent boronic acid incorporation ratio was consistent with the amount of APBA (**2**) taken for the coupling reaction and the results have demonstrated the reliability of a new method for preparation of affinity agent (**3**).[15]

Each of the reagent solution (**3**) in taurine buffer (Table 2, entries: 1–6) was tested for performance in the measurement of GHb using three assay controls (low, medium, and high). Interestingly, with the increase in percent boronic acid incorporation, the affinity agent (**3**) read each of the controls higher. The reagent (**3**) prepared with 0.309 equiv of APBA (**2**) (entry 1) read the three controls, 6.9, 11.7, and 16.4, at low end of the range and outside of assay specification. Whereas the agent (**3**) prepared with 0.619 equiv of APBA (**2**) (entry 6) read all three controls, 9.0, 13.4, and 17.5, at higher end of the range and outside of specification. However, the affinity reagent (**3**) prepared with 0.475 equiv of APBA (**2**) (entry 4) gave best performance results with in the range and close to the target values for low, medium, and high controls, 8.1, 12.5, and 16.7, respectively, as desired. These results clearly demonstrated that the performance of affinity agent (**3**) is critically dependent on boronic acid incorporation ratio, and must be controlled in order to achieve good precision in GHb measurement.

We then studied the effect of affinity agent (**3**) concentration in taurine buffer (Table 3) on GHb measurement. Accordingly, the powdered agent (**3**), which was prepared using the PACA (**1**) with molecular weight of 225 kDa and 0.412 equiv of APBA (**2**), was dissolved in taurine buffer and the concentration was adjusted to four different levels, 0.36, 0.39, 0.45, and 0.49 (entries: 1–4) based on the UV absorbance. All four reagent solutions (entries: 1–4) were tested for performance in the

Table 2. Preparation of affinity agent (**3**) using 225 kDa MW PACA (**1**) with different ratios of APBA (**2**) and measurement of GHb

Entry	Ratio of reagents PACA (**1**):APBA (**2**)[a,b] (equiv)	Affinity agent (**3**)		GHb measurement[d]		
		UV (252 nm)	IR (%)[c]	Low	Medium	High
1	1.0:0.309	0.38	24–27	6.9	11.7	16.4
2	1.0:0.412	0.37	33–35	7.4	12.1	16.4
3	1.0:0.455	0.37	35–40	7.8	12.3	16.5
4	1.0:0.475	0.36	37–40	8.1	12.5	16.7
5	1.0:0.516	0.37	40–45	8.6	12.9	17.1
6	1.0:0.619	0.37	42–49	9.0	13.4	17.5

[a] PACA (**1**) sodium salt, 20% solids in water with MW of 225 kDa and the APBA (**2**) was present as its hemisulfate salt.
[b] In all entries (1–6), 1.94 equiv of EDAC was used based on APBA hemisulfate (**2**).
[c] The percent boronic acid incorporation ratio (IR) was calculated based on UV absorbance and elemental analysis.
[d] GHb assay was performed using three controls, low, medium, and high [see footnote (d) in Table 1].

Table 3. Preparation of affinity agent (**3**) with different concentrations in taurine buffer and GHb measurement

Entry	Ratio of reagents PACA (**1**):APBA (**2**) (equiv)	Affinity reagent (**3**)		GHb measurement[b]		
		IR (%)[a]	UV (252 nm)	Low	Medium	High
1	1.0:0.412	33–35	0.36	7.3	11.6	16.2
2	1.0:0.412	33–35	0.39	7.4	12.1	16.4
3	1.0:0.412	33–35	0.45	8.1	12.3	16.6
4	1.0:0.412	33–35	0.49	8.1	12.5	16.7

[a] Average boronic acid incorporation ratio (IR) in reagent (**3**) was determined based UV and elemental analysis.
[b] GHb assay was performed using three controls, low, medium, high [see footnote (d) in Table 1].

measurement of GHb using the three controls and the results are depicted in Table 3. It was found that with increase in concentration of affinity agent (**3**) in taurine buffer, the reading of controls changed from below specification range to closer to the target values. These results indicated that in addition to the boronic acid incorporation ratio, the concentration of affinity agent (**3**) in buffer also plays an important role in GHb measurement. Based on this study, the polyanionic affinity agent (**3**) prepared using poly(acrylic acid) (**1**) with 225 kDa molecular weight was found to be effective for GHb measurement. The ratio of 3-aminophenylboronic acid hemisulfate (**3**) and poly(acrylic acid) (**1**) is critically important to achieve the appropriate boronic acid incorporation and 0.48 equiv was determined to be an optimal ratio. Additionally, the concentration of affinity agent (**3**) in taurine buffer also impacts glycated hemoglobin measurement and 0.36–0.38 range at 252 nm needs to be maintained to achieve consistent performance results.

3. Conclusion

An efficient method was developed for the preparation of polyanionic affinity agent (**3**), the key component in the measurement of glycated hemoglobin (GHb). The effect of the molecular weight of poly(acrylic acid) (**1**) on GHb measurement was studied and it was found that the affinity agent (**3**) prepared using 225 kDa poly(acrylic acid) (**1**) gave the best performance results. The role of boronic acid incorporation ratio in the affinity agent (**3**) and its concentration in taurine buffer was evaluated for GHb measurement, and discovered that both factors play a critical role in its performance. Evaluation of various key features of this polymeric affinity agent (**3**) and determination of their correlation to the performance emphasize the importance of a reliable method for its preparation. The new method described for the preparation of affinity agent (**3**) is efficient and affords a consistent product. Utilization of a reliable, quality polyanionic affinity agent (**3**) is critically important in the measurement of glycated hemoglobin values with accuracy and high precision, which are useful for diagnosis and management of diabetes.

4. Experimental

4.1. General methods and materials

Poly(acrylic acid) (**1**) with an average molecular weight of 225 kDa was purchased from Polyscience, Inc., (Warrington, PA) as its sodium salt and 20% solid in water. All other chemicals were purchased from Aldrich Chemical Co., (Milwaukee, WI). MES buffer (50 mM, pH 5.5) was prepared from 22.4 g of 2-[*N*-morpholino]ethanesulfonic acid (MES) in 4.0 L of water and the pH was adjusted to 5.5 using 1 N sodium hydroxide solution. Taurine buffer (50 mM, pH 9.0) was prepared from 25 g of taurine in 4.0 L of water (4.0 L) and the pH was adjusted to 9.0 using 10 N sodium hydroxide solution.

The three GHb assay controls are, low (target value: 8.3 and range: 7.6–9.0), medium (target value: 12.6 and range: 11.9–13.3) and high control (target value: 17.0 and range: 16.3–17.7). The assay controls and calibrators were prepared from an admixture of normal human hemoglobin (Hb) with the processed human hemoglobin (Hb). The processed human hemoglobin was prepared based on in vitro glycosylation of normal hemoglobin by incubating with glucose followed by reduction using sodium cyanoborohydride.[8]

4.2. General procedure for preparation of polyanionic affinity agent (3)

In 6.0 L beaker equipped with mechanical stirrer, thermometer and pH probe, the poly(acrylic acid) sodium salt (PACA, **1**, average molecular weight: 225 kDa, 200 g of sodium salt, 20% solids in water, 425.4 mmol, based on the acrylic acid sodium salt) was taken and diluted with water (3.4 L). To this solution, 3-aminophenylboronic acid hemisulfate (APBA hemisulfate, **2**, 38.5 g, 206.9 mmol, 0.486 equiv) was added and the pH of the mixture was adjusted to about 6.1 using 10 N sodium hydroxide solution. In a separate 2.0 L beaker, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC, 77.0 g, 401.7 mmol, 0.94 equiv) was dissolved in water (900 mL) and the pH of the solution was quickly adjusted to 5.42 with 1.0 N hydrochloric acid. The EDAC–water solution was then added slowly to the reaction mixture using an addition funnel with vigorous stirring, at room temperature (21–23 °C) over a period of about 15 min. An aliquot was taken and analyzed by HPLC (YMC-AQ-ODS C18, RP column, MeCN:0.05% aqueous trifluoroacetic acid/gradient system: 5:95 to 95:5 in 20 min, 1.0 mL/min at 254 nm) and found that the APBA (**2**) was consumed >95%. The resulting clear mixture was stirred for an additional 1 h at room temperature. The reaction mixture was then acidified by slow addition of 1.0 N hydrochloric acid (400 mL) over a 15 min period, to adjust the pH of the mixture to about 1.8. The resulting white precipitate was stirred for an additional 10–15 min and filtered. The white solid was washed thoroughly four times with water (4 × 800 mL) and the resulting solid material (350 g) was dried in an oven at 35 °C temperature under vacuum. The material was mixed periodically and after drying for 72 h, the solid was powdered to afford 57.6 g of affinity agent (**3**) in 95% yield as a colorless (white) powder. Analysis: Found, C, 52.68; H, 5.17; B, 3.34; N, 6.68, boronic acid incorporation ratio: 38%.

In a 4 L beaker equipped with a mechanical stirrer and a pH probe, the affinity agent powder (**3**, 22.0 g) was taken and taurine buffer (2.0 L, 50 mM, pH 9.0) was added at room temperature (21–23 °C). The mixture was gently stirred for 6 h at room temperature and during this period, the pH of the mixture was adjusted two times using 10 N sodium hydroxide solution and it was maintained between 8.5 and 9.0 pH. After stirring the mixture for 6 h, the pH was finally adjusted to 9.0 using 10 N sodium hydroxide solution, as needed, and the mixture was diluted with taurine (200 mL). The solution

was filtered to afford the polyanionic affinity reagent (**3**) as a colorless solution. UV absorbance: 0.37 at 252 nm, boronic acid incorporation ratio: 39%.

4.3. Preparation of affinity reagent (3) using different molecular weight PACA (1)

(a) *30 kDa PACA*: 12.3 g of PACA sodium salt (**1**, 40% solids in water, 52.32 mmol) and 4.72 g of APBA (**2**, 25.38 mmol, 0.485 equiv) using 9.44 g of EDAC (49.24 mmol, 0.94 equiv) gave 5.48 g of powdered agent (**3**). Analysis: Found, C, 54.70; H, 5.42; B, 3.34; N, 6.24, boronic acid incorporation ratio: 38%. Reagent (**3**) in taurine buffer, UV absorbance: 0.37 at 252 nm, boronic acid incorporation ratio: 39%.

(b) *100 kDa PACA*: 10.75 g of PACA (**1**, 35% solids in water, 52.2 mmol) and 4.72 g of APBA (**2**, 25.38 mmol, 0.485 equiv) and 9.44 g of EDAC (49.24 mmol, 0.94 equiv) gave 6.4 g of powdered agent (**3**). Analysis: Found, C, 55.22; H, 5.38; B, 3.40; N, 6.24, boronic acid incorporation ratio: 38%. Reagent (**3**) in taurine buffer, UV absorbance: 0.37 at 252 nm, boronic acid incorporation ratio: 37%.

(c) *240 kDa PACA*: 15.0 g of PACA (**1**, 25% solids in water, 52.2 mmol) and 4.72 g of APBA (**2**, 25.38 mmol, 0.485 equiv) and 9.44 g of EDAC (49.24 mmol, 0.94 equiv) gave 6.6 g of powdered agent (**3**). Analysis: Found, C, 53.70; H, 5.56; B, 2.94; N, 6.48, boronic acid incorporation ratio: 38%. Reagent (**3**) in taurine buffer, UV absorbance: 0.38 at 252 nm, boronic acid incorporation ratio: 37%.

(d) *450 kDa PACA*: From 3.76 g of PACA (**1**, 52.2 mmol) and 4.72 g of APBA acid (**2**) (25.38 mmol, 0.485 equiv) and 9.44 g of EDAC (49.24 mmol, 0.94 equiv) gave 6.4 g of powdered agent (**3**). Analysis: Found, C, 53.27; H, 5.50; B, 2.99; N, 6.46, boronic acid incorporation ratio: 38%. Reagent (**3**) in taurine buffer, UV absorbance: 0.38 at 252 nm, boronic acid incorporation ratio: 38%.

4.4. Preparation of affinity reagent (3) using different ratios of APBA hemisulfate (2):

(a) *0.309 equiv*: 24.5 g of PACA sodium salt (**1**, 225 kDa, 20% solids in water, 52.1 mmol) and 3.0 g of APBA hemisulfate (**2**, 16.13 mmol, 0.309 equiv) using 6.0 g of EDAC (31.30 mmol, 0.60 equiv) gave 5.3 g of affinity agent (**3**) powder. Analysis: Found, C, 54.18; H, 5.48; B, 2.31; N, 4.91. Reagent (**3**) in taurine buffer (pH 9.0), UV absorbance: 0.38 at 252 nm. Boronic acid incorporation ratio: 24–27%.

(b) *0.412 equiv*: 98.0 g of PACA (**1**, 208.4 mmol) and 16.0 g of APBA (**2**, 86.04 mmol, 0.412 equiv) using 32.0 g of EDAC (172.04 mmol, 0.83 equiv) gave 23.9 g of affinity agent (**3**) powder. Analysis: Found, C, 54.60; H, 5.63; B, 3.17; N, 6.16. Reagent (**3**) in taurine buffer with four concentrations, UV: 0.36, 0.39, 0.45, and 0.49 at 252 nm. Incorporation ratio: 33–35%.

(c) *0.455 equiv*: 24.5 g of PACA (**1**, 52.1 mmol) and 4.4 g of APBA (**2**, 23.66 mmol, 0.455 equiv) using 8.8 g of EDAC (45.91 mmol, 0.88 equiv) gave 6.4 g of affinity agent (**3**) powder. Reagent (**3**) in taurine buffer, UV: 0.37 at 252 nm. Incorporation ratio: 35–40%.

(d) *0.475 equiv*: 24.5 g of PACA (**1**, 52.1 mmol) and 4.6 g of APBA (**2**, 24.71 mmol, 0.48 equiv) using 9.2 g of EDAC (48.0 mmol, 0.92 equiv) gave 6.6 g of affinity agent (**3**) powder. Reagent (**3**) in taurine buffer, UV: 0.36 at 252 nm. Incorporation ratio: 37–40%.

(e) *0.515 equiv*: 24.5 g of PACA (**1**, 52.1 mmol) and 5.0 g of APBA (**2**, 26.88 mmol, 0.52 equiv) using 10.0 g of EDAC (52.16 mmol, 1.0 equiv) gave 6.7 g of affinity agent (**3**) powder. Reagent (**3**) solution in taurine buffer, UV: 0.37. Boronic acid IR: 40–45%.

(f) *0.619 equiv*: 24.5 g of PACA (**1**, 52.1 mmol) and 6.0 g of APBA (**2**, 32.25 mmol, 0.62 equiv) using 12.0 g of EDAC (62.59 mmol, 1.20 equiv) gave 7.5 g of affinity agent (**3**) powder. Reagent (**3**) in taurine buffer, UV: 0.37 at 252 nm. Incorporation ratio: 40–49%.

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15. The boronic acid incorporation ratio (IR) in affinity agent (**3**) was determined based on two methods, (a) UV absorbance and (b) elemental analysis. UV absorbance of reagent (**3**) solution was measured at 252 nm and the extinction coefficient (ε) of 7580 M^{-1} cm^{-1} was used for calculation. The extinction coefficient (ε) was determined based on two model conjugates, 3-(dihydroxyboryl)acetamide (derivative of acetic acid) and 5-{[3-(dihydroxy boryl)phenyl]amino}-5-oxo-3-phenylpentanoic acid (derivative of glutaric acid) in taurine buffer (50 mM, pH 9.0) at 252 nm. The elemental analysis method for determination of boronic acid incorporation ratio was based on observed values versus the theoretical values of a statistical mixture of acrylic acid and its 3-aminophenylboronic acid derivative, 3-(dihydroxyboryl)acrylamide.