Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 16 (2006) 1324–1328
Bioorganic & Medicinal Chemistry Letters

Chemiluminescent acridinium-9-carboxamide boronic acid probes: Application to a homogeneous glycated hemoglobin assay

Maciej Adamczyk,* Yon-Yih Chen, Donald D. Johnson, Phillip G. Mattingly, Jeffrey A. Moore, You Pan and Rajarathnam E. Reddy

Abbott Laboratories, Diagnostics Division, 100 Abbott Park Road, AP20, Abbott Park, IL 60064-6016, USA

Received 3 October 2005; revised 10 November 2005; accepted 15 November 2005
Available online 15 December 2005

Abstract—Chemiluminescent acridinium-9-carboxamide probes containing 1, 3, 9, and 27 phenylboronic acids were prepared and their chemiluminescent properties evaluated. The relative chemiluminescent signal from the probes varied from 4 to 0.83×10^{19} counts/mol across the series, while the apparent affinity of the probes for the diabetes marker glycated hemoglobin increased from 211 to 0.43 μM. The dose-dependent modulation of the chemiluminescent intensity of the probes upon binding was used to demonstrate a homogeneous assay for glycated hemoglobin.

The signal from chemiluminescent acridinium-9-carboxamide-modified ligands can be specifically quenched/modulated upon non-covalent association with binding proteins specific for the ligand. The ligand–binding protein pairs investigated thus far have included biotin–avidin,[1] folic acid–folate binding protein,[2] and vitamin B_{12}—intrinsic factor.[3] In each case, the degree of signal modulation was dose-dependent and sufficient to demonstrate a competitive homogeneous assay for the ligand of interest.

In this communication, we report a homogeneous chemiluminescent assay for glycated hemoglobin, a diagnostic marker for which no innate binding protein–ligand pairing is available.

Glycated hemoglobin (GHb) is the marker of choice in assessing patient glycemic status during the diagnosis and treatment of diabetes.[4,5] The non-enzymatic reaction of glucose and hemoglobin (Hb) primarily forms a Schiff base with the β-chain, amino terminal valine that subsequently undergoes an Amadori rearrangement to give a stable *N*-(1-deoxy-D-fructos-1-yl)amino derivative (designated HbA_{1c}) (Fig. 1). Other minor glycation products are also formed and contribute to the total

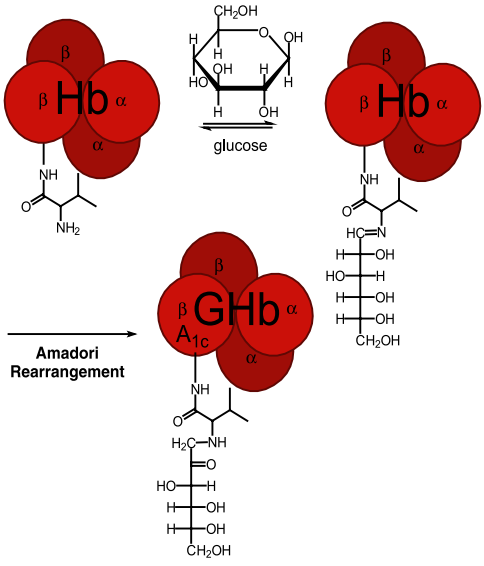


Figure 1. Formation of glycated hemoglobin (GHb) via the Amadori rearrangement.

%GHb. Since the half-life of the hemoglobin molecule in vivo is relatively long, the degree of glycation reflects the time-averaged concentration of glucose over the previous 6–8 weeks.

The current trends in assaying this marker have been recently reviewed.[4] Among the most useful assays are

Keywords: Chemiluminescence; Homogeneous assay; Multivalent ligand; Glycated hemoglobin; Diabetes marker; Boronic acid.
* Corresponding author. Tel.: +1 847 937 0225; fax: +1 847 938 8927; e-mail: maciej.adamczyk@abbott.com

0960-894X/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2005.11.062

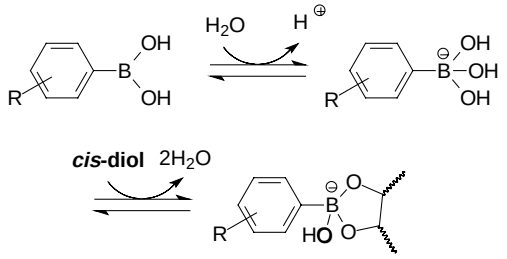
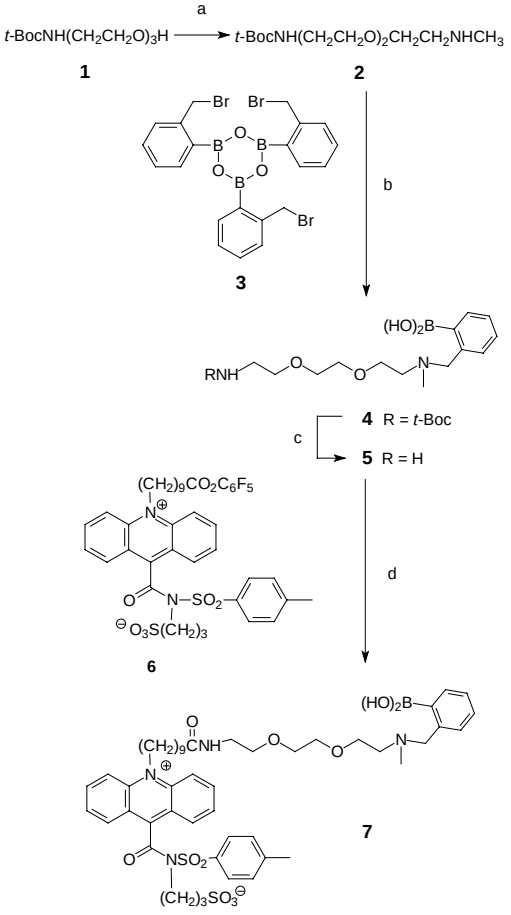


Figure 2. Specific interaction of arylboronic acids with *cis*-diols.

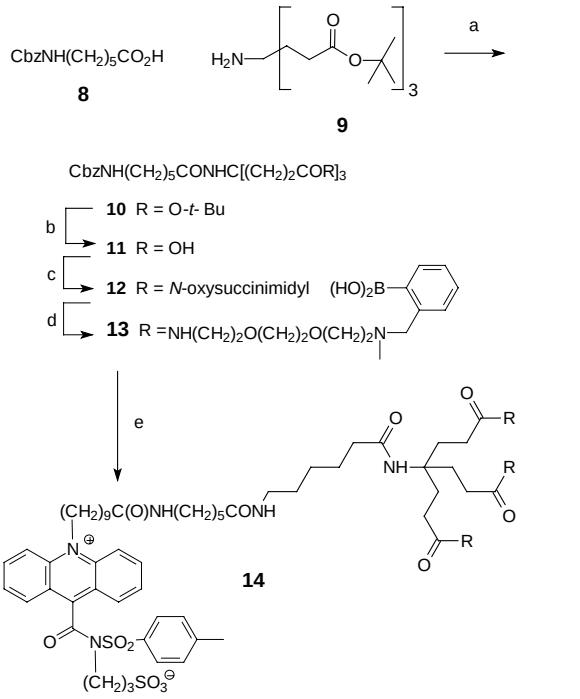
those that report total %GHb based on the specific reaction of aryl boronic acids with the *cis*-diols of the glycated molecule (Fig. 2).

The stability of the boronate ester is rather low in aqueous media ($\log K \approx 3$) and typically is augmented by the multivalent interactions provided by polymeric[6] or dendritic boronic acids[7] (see also any of the several excellent reviews on boronic acid carbohydrate sensors[8–10]).

To demonstrate chemiluminescent signal modulation in the detection of glycated hemoglobin, a series of acridi-



Scheme 1. Preparation of mono-boronic acid chemiluminescent probe **7**. Reagents and conditions: (a) i—MsCl, Et_3N, toluene, 0 °C, 2 h; ii—methylamine, 7 d, 68%; (b) **3**, K_2CO_3, AcCN, 69%; (c) i—TFA, CH_2Cl_2, 15 min; (d) **6**, DMF/buffer (pH 8), 2 h, 50%.

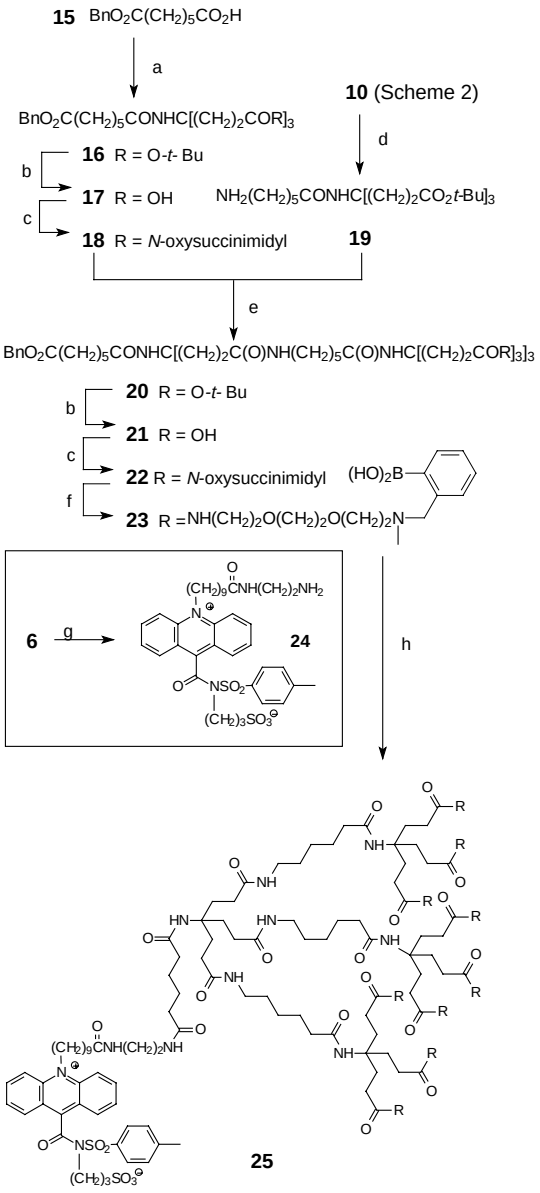


Scheme 2. Preparation of trivalent boronic acid chemiluminescent probe **14**. Reagents and conditions: (a) i—oxalyl chloride, CH_2Cl_2, DMF; ii—Behera's amine *tert*-butyl ester **9**, Et_3N, CH_2Cl_2, 1 h, 70%; (b) TFA, CH_2Cl_2, 2 h, 88%; (c) *N*-hydroxysuccinimide, EDAC, DMF, 14 h, 62%; (d) **5**, Et_3N, DMF, 14 h, 79%; (e) i—HBr/AcOH, 45 min, 83%; ii—**6**, Et_3N, DMF, 14 h, 86%.

nium-9-carboxamide boronic acid reagents were prepared containing from 1 to 27 boronic acid groups, with the aim of identifying a reagent with the minimum diol 'affinity' sufficient for an assay. The increased stability and operable pH range offered by *o*-aminomethyl arylboronic acids in the formation of the boronate ester[11] prompted the choice of compound **5** as the common element for incorporation into the target reagents.

Thus, as shown in Scheme 1,[12] alcohol **1**[13] was converted to the methylamine linker **2**, and then alkylated by the *o*-bromomethylphenyl boronic acid anhydride **3**[14,15] to afford the *t*-Boc-protected intermediate **4**. Subsequent trifluoroacetic acid deprotection to **5** and acylation with acridinium-9-carboxamide pentafluorophenyl active ester **6**[2] gave the desired mono-boronic acid chemiluminescent probe **7**.

Chemiluminescent arborol probes **14**, **25**, and **35** bearing 3, 9, and 27 boronic acid surface groups, respectively, were prepared as shown in Schemes 2–4. The *tert*-butyl ester of Behera's amine[16] (**9**, Scheme 2) served as the initial AB_3 branching unit. The focal point was extended by the addition of a 6-(Cbz-amino)caproic acid linker to give **10**. Acid deprotection, activation, and coupling to boronic acid **5** afforded **13**. Removal of the Cbz-protecting group by hydrogenolysis and acylation with acridinium active ester **6** completed the preparation of the trivalent reagent **14**. Preparation of the 9-mer **25**



Scheme 3. Preparation of nonavalent-boronic acid chemiluminescent probe **25**. Reagents and conditions: (a) i—oxalyl chloride, CH_2Cl_2, DMF; ii—Behera's amine *tert*-butyl ester **9**, Et_3N, CH_2Cl_2, 1 h, 86%; (b) TFA, CH_2Cl_2, 2 h, 72–84%; (c) *N*-hydroxysuccinimide, EDAC, DMF, 14 h, 63–74%; (d) H_2, Pd/C, MeOH, 100%; (e) **19**, Et_3N, DMF, 14 h, 79%; (f) **5**, Et_3N, DMF, 14 h, 86%; (g) $NH_2CH_2CH_2NH_2$, DMF/buffer (pH 8), 1 h, 45%; (h) i—HBr/AcOH, 45 min, 80%; ii—pentafluorophenyl trifluoroacetate, pyridine, DMF, 14 h, 57%; iii—**24**, Et_3N, DMF, 14 h, 48%.

(Scheme 3) and the 27-mer **35** (Scheme 4) proceeded similarly, using the advanced intermediates from the preceding schemes where possible.

The chemiluminescence properties of each probe are shown in Figure 3. The chemiluminescent signal gradually dropped from 4×10^{19} to 8.3×10^{18} counts/mol as the number of boronic acids increased across the series, while the emission profile for each displayed the 'flash' kinetics typical for the unsubstituted acridinium-9-carboxamide.

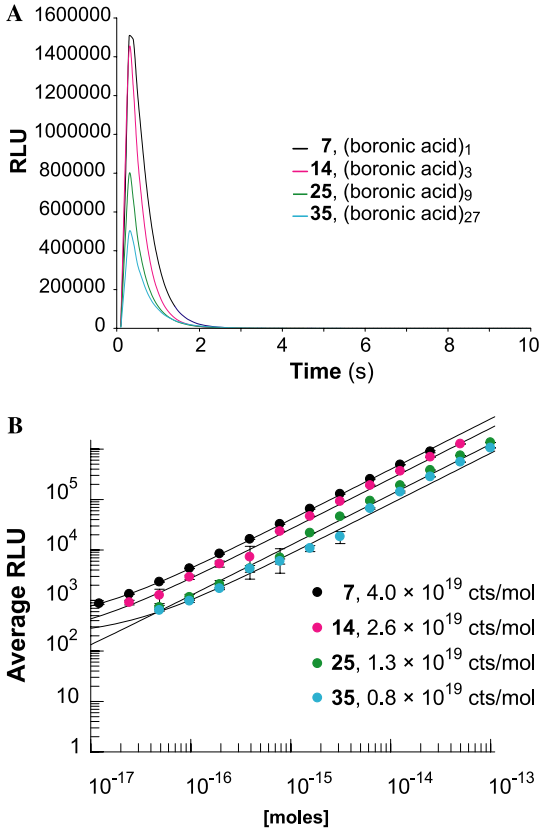
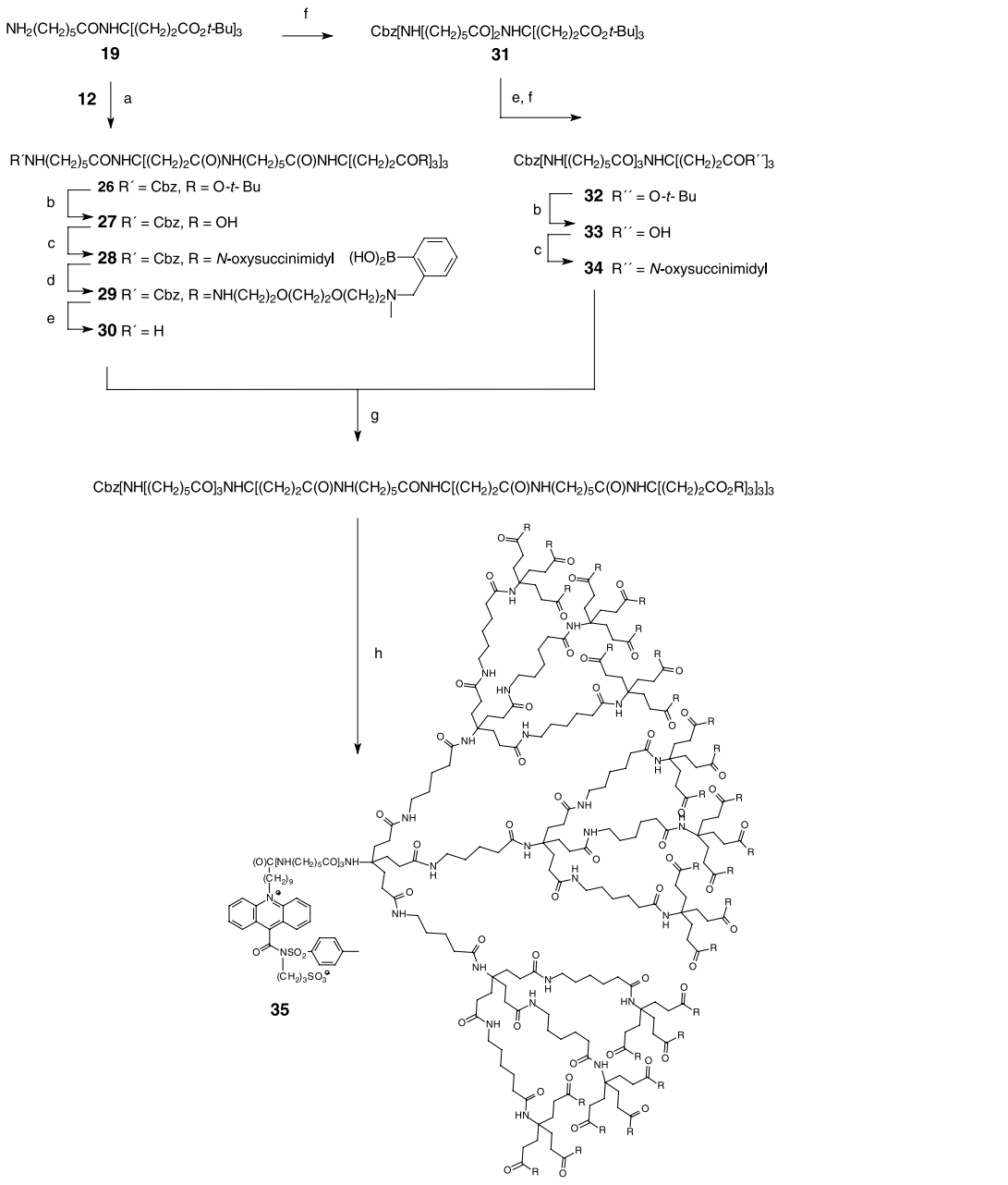


Figure 3. Chemiluminescence of boronic acid probes. (A) Chemiluminescence emission profiles. (B) Dilution linearity. Conditions: aliquots (25 μL) of each sample were triggered using 200 μL of triggering solution (0.35 N NaOH, 1.32% H_2O_2, 0.09% DTPA, and 2% Triton X-100). The chemiluminescent response from each sample was integrated over a 10s window.

The chemiluminescent response of the boronic acid reagents **7**, **14**, **25**, and **35** was next evaluated in the presence of stabilized human glycated hemoglobin (azido-methemoglobin, 40% glycation by vendor assay) and 'non-glycated' hemoglobin (azido-methemoglobin, 5% glycation by vendor assay) both obtained from American Biological Technology, Inc. (Fig. 4). The chemiluminescent signal was recorded from samples containing a fixed concentration of the chemiluminescent reagent (0.5 nM) and either glycated or non-glycated hemoglobin upon addition of basic hydrogen peroxide. Total hemoglobin concentration was determined using a commercial kit following the manufacturer's protocol (Sigma Total Hemoglobin). Representative raw data using the trivalent boronic acid **14** are seen in Figure 4A.

The degree of signal modulation was dependent on the total amount of hemoglobin present. Similar quenching effects have been noted with fluorescent indicators in the presence of hemoglobin.[17] At each concentration, the chemiluminescent signal from the glycated hemoglobin was lower than that of the non-glycated one indicating that upon formation of the boronate ester the signal is further quenched. The concentration-dependent quenching specific to the formation of the boronic



Scheme 4. Preparation of 27-valent boronic acid chemiluminescent probe **35**. Reagents and conditions: (a) Et_3N, DMF, 14 h; (b) TFA, CH_2Cl_2, 2 h, 72–85%; (c) *N*-hydroxysuccinimide, EDAC, DMF, 14 h, 63–72%; (d) **5**, Et_3N, DMF, 14 h, 75%; (e) HBr/HOAc, 2 h, 89%; (f) 6-(Cbz-amino)caproic acid **8**, HOBt, EDAC, CH_2Cl_2, 14 h, 72–87%; (g) H_2 Pd/C, MeOH, 99%; (h) Et_3N, DMF, 14 h, 50%; (i) i—HBr/HOAc, 45 min, 72%; ii—**6**, DMF/buffer (pH 8), 5 h, 58%.

acid-glycated hemoglobin complex is presented in Figure 4B.

The effect of multivalency is clearly evident. The apparent binding constants derived from the curves indicate a 500-fold improvement on going from the mono-boronic acid **7** to the 27-mer **35**. It is also interesting to note that at total Hb concentrations below 10 μM, the chemiluminescent signal from the weakest binder, mono-boronic acid **7** is not quenched, while **25** and **35** just reach their maximal signal attenuation at 10 μM. The conclusions from this observation are twofold. First, at concentrations below 10 μM Hb non-specific quenching of the acridinium chemiluminescent signal by hemoglobin is absent. Second, the nonavalent boronic acid **25** had sufficient affinity for GHb to operate in that concentration range.

Testing of these observations with human whole blood samples with established values for glycated hemoglobin (as $\%HbA_{1c}$) resulted in the dose–response curve depicted in Figure 5.

The five samples were first treated with potassium ferricyanide (130 mol %) to convert hemoglobin to methemoglobin (analogous to the stabilized hemoglobin used above) and then diluted in buffer to give a total hemoglobin concentration of 10 μM. The data shown

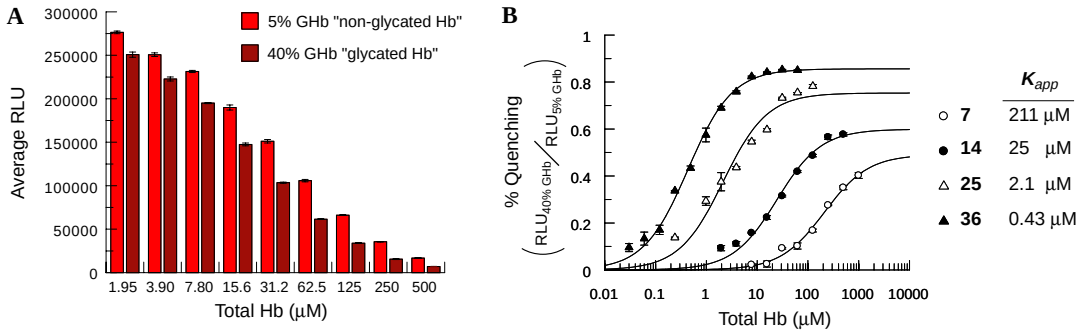


Figure 4. Chemiluminescent signal attenuation by 'non-glycated' and 'glycated' hemoglobin. (A) Illustrative raw data using probe **14**. (B) Apparent affinity of probes **7**, **14**, **25**, and **36**. Conditions: non-glycated hemoglobin (ABT, Inc.) contained the normal physiological level of glycation (5%), while the glycated samples contained a high level of modification (40%). Solutions containing the chemiluminescent boronic acids (0.5 nM) and hemoglobin were prepared in buffer (10 mM HEPES, 2 mM EDTA, and 0.1% Tween 20, pH 7.3). Aliquots (25 μL) of each sample were triggered (200 μL, 0.35 N NaOH, 1.32% H_2O_2, 0.09% DTPA, and 2% Triton X-100) and the chemiluminescent response from each sample was integrated over a 2s window. Data points represent the average of triplicate values.

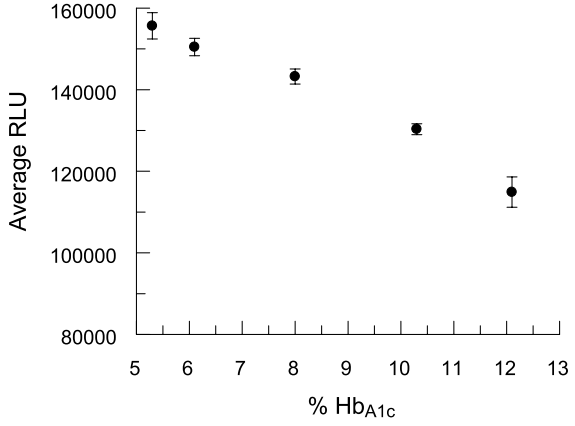


Figure 5. Glycated hemoglobin dose–response curve with chemiluminescent boronic acid probe **25**. Conditions: whole blood samples (Trimar) were treated with $K_3Fe(CN)_6$ (130 mol %) and diluted to a final concentration of 10 μM along with **25** (0.5 nM) in buffer (10 mM HEPES, 2 mM EDTA, and 0.1% Tween 20, pH 7.4). Aliquots (25 μL) of each sample were triggered (200 μL, 0.35 N NaOH, 1.32% H_2O_2, 0.09% DTPA, and 2% Triton X-100.) The chemiluminescent response from each sample was subsequently integrated over a 2s window.

here represent the average of triplicate reads for triplicate samples (9 data points/sample). The response over the clinically relevant %HbA_{1c} range of 5–12% showed good linearity ($R = 0.9902$) and slope, thus demonstrating the foundation for a homogeneous assay for glycated hemoglobin.

Further studies to expand the utility of homogeneous chemiluminescent assays are in progress.

References and notes

1. Adamczyk, M.; Mattingly, P. G.; Moore, J. A.; Pan, Y. *Org. Lett.* **2003**, *5*, 3779.
2. Adamczyk, M.; Fino, J. R.; Mattingly, P. G.; Moore, J. A.; Pan, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2313.
3. Adamczyk, M.; Johnson, D. D.; Mattingly, P. G.; Moore, J. A.; Pan, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3917.
4. Blincko, S.; Edwards, R. *Clin. Lab.* **2001**, *47*, 377.
5. Krishnamurti, U.; Steffes, M. W. *Clin. Chem.* **2001**, *47*, 1157.
6. Wilson, D. H.; Bogacz, J. P.; Forsythe, C. M.; Turk, P. J.; Lane, T. L.; Gates, R. C.; Brandt, D. R. *Clin. Chem.* **1993**, *39*, 2090.
7. James, T. D.; Shimori, H.; Takeuchi, M.; Shinkai, S. *Chem. Commun.* **1996**, 705.
8. Hartley, J. H.; James, T. D.; Ward, C. J. *J. Chem. Soc., Perkin Trans. 1* **2000**, 3155.
9. James, T. D.; Shinkai, S. *Top. Curr. Chem.* **2002**, *218*, 159.
10. Wang, W.; Gao, X.; Wang, B. *Curr. Org. Chem.* **2002**, *6*, 1285.
11. Wulff, G. *Pure Appl. Chem.* **1982**, *54*, 2093.
12. All new compounds were purified by reverse-phase HPLC to greater than 95% purity and yielded the expected *m/z* by electrospray mass spectrometry.
13. Lebeau, L.; Oudet, P.; Mioskowski, C. *Helv. Chim. Acta* **1991**, *74*, 1697.
14. Hawkins, R. T.; Snyder, H. R. *J. Am. Chem. Soc.* **1960**, *82*, 3863.
15. Cooper, C. R.; James, T. D. *J. Chem. Soc., Perkin Trans. 1* **2000**, 963.
16. Newkome, G. R.; Behera, R. K.; Moorefield, C. N.; Baker, G. R. *J. Org. Chem.* **1991**, *56*, 7162.
17. Blincko, S.; Edwards, R. *Ann. Clin. Biochem.* **1998**, *35*, 140.