OMB No. 0925-0195
Expiration Date 2/28/98

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Quantitative, Rapid Sensor for Glycosylated Hemoglobins PSC-NIH (1)

2. SOLICITATION NO. PHS 97-2

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

3a. NAME *(Last, first, middle)*
Van Wagenen, Richard A.

3b. DEGREE(S)
B.S. Ph.D.

3c. SOCIAL SECURITY NO. *(Provide on Personal Data Page)*

3d. POSITION TITLE
Vice President for Research & Development

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Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093
BITNET/INTERNET Address:

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 801-583-9301
FAX: 801-583-4463

4. HUMAN SUBJECTS
☒ NO ☐ YES
4a. If "yes," Exemption no. or IRB approval date
☐ Full IRB or ☐ Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS
☒ NO ☐ YES
5a. If "Yes," IACUC approval date
5b. Animal welfare assurance no.

6. DATES OF PROJECT PERIOD
From: September 30, 1998 *Through:* March 30, 1999

7. COSTS REQUESTED
7a. Direct Costs: $ 70,037
7b. Total Costs: $ 98,559

8. PERFORMANCE SITES *(Organizations and addresses)*
Protein Solutions, Inc.
391 G Chipeta Way, Suite 320
Salt Lake City, UT 84108

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84518-0093

10. ENTITY IDENTIFICATION NUMBER
Fed. Tax # 87-045-1813

Congressional District: 2

11. SMALL BUSINESS CERTIFICATION
☒ Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

12. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages _____ of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ☒ YES ☐ NO

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: J. D. Andrade
Title: President and CEO
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15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*
Richard A. Van Wagenen
DATE: 12-12-97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*
DATE: 12-12-97

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093 Phone: 801-583-9301

YEAR FIRM FOUNDED	NO. OF EMPLOYEES *(include all affiliates)*
1987	5

TITLE OF APPLICATION

Quantitative, Rapid Sensor for Glycosylated Hemoglobins

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
R. Van Wagenen, Ph.D.	Protein Solutions, Inc.	Principal Investigator
C.-Y. Wang, Ph.D.	" " "	Research Scientist
R. Scheer, Ph.D.	" " "	Research Scientist
Q. Luo	Now at the University of Utah	Post Doc (to be hired)
J. D. Andrade, Ph.D.	" " "	Advisor

ABSTRACT OF RESEARCH PLAN: State the application's broad, long-term objectives and specific aims, making reference to the health-relatedness of the project. Describe concisely the research design and methods for achieving these goals and discuss the potential of the research for technological innovation. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. ***Therefore, do not include proprietary or confidential information.*** DO NOT EXCEED 200 WORDS.

Glycated hemoglobin (gHb) in red blood cells provides a history of glucose concentration and activity during the life time of that red blood cell. Therefore, monitoring of glycated hemoglobin has become an important means of objectively assessing the glycemic state in diabetics. There are a number of problems with existing methods for the assay of gHb. Chemical colorimetric reactions, including the so called fructosamine assay, do not have the needed specificity. There are also problems with immunoassays in that the full glycosylated hemoglobin profile is difficult and/or expensive to obtain. The method of choice is generally high resolution chromatography or electrophoresis. These techniques are difficult to miniaturize. Labor and equipment are intensive and therefore expensive. We propose to develop a thin layer chromatography based device to permit high resolution separation, visualization, and quantification of gHb without the need for instruments or readout devices. This simple device is disposable, inexpensive, reliable, and easy to use, and will permit high resolution separation coupled with direct visualization and quantification. It will utilize a bi-assay chromatographic system involving both cation and affinity chromatography and a unique bioluminescence-based visualization and quantification technology.

Provide key words (8 maximum) to identify the research or technology.

hemoglobin, diabetes, glycohemoglobin, glucose, fructosamine, luciferase

Provide a brief summary of the potential commercial applications of the research.

A simple gHb measurement device would be of interest to the 15 million Americans who have Type II diabetes (half of them undiagnosed and, therefore, largely not aware of their problem and its potential consequences). Assuming the device could sell in the $ 5.00-10.00 range and be used 2-10 times per year, the market in the U.S. alone, is estimated to be approximately $100 million per year.

A. Specific Aims

The general goal and aim of this Phase I project is to assess the feasibility of utilizing a thin layer chromatography-based device for the high resolution separation of hemoglobins. A further aim and objective (in Phase II) is to visualize the separated protein using an ATP dependent enzyme blot followed by an ATP specific firefly luciferase blot. This method utilizes a unique gradient technology which allows the visualization and quantitation of each of the individual hemoglobin bands. The visualization and quantitation technologies are the basis of other on going projects; their application to the gHb device will be more fully discussed in the Phase II application.

We propose to:

1. evaluate means of separating and lysing red blood cells, permitting hemoglobin to be released and transported to the front end ion exchange portion of the device.

2. develop a thin layer cationic exchange device for the capillarity driven, pH and/or ionic strength gradient modulated, separation of hemoglobins. This task will involve the evaluation of a number of commercial cationic exchangers and the determination of the optimum pH and ionic strength gradient conditions for maximal separation and resolution.

3. evaluate and optimize an affinity chromatography second stage to more fully and completely separate the gHb bands. The affinity chromatography second stage will assess the feasibility of a boronic acid affinity technique.

4. begin the integration of the four basic components of the device: red cell separation and lysis, hemoglobin transport and delivery, ion exchange separation, and affinity separation. Further optimization of separation and resolution will involve optimization of device dimensions, volumes, and separation times.

5. evaluate an ATP—or NAD(P)H—based labeling system to permit quantitative detection using the highly sensitive and specific firefly or bacterial luciferase technique. Most of this work will be done in Phase II.

The goal is to establish the feasibility of a thin layer device for gHb separation, detection, and quantitation.

The main objectives of the Phase II application will be to more fully define and enhance the device, especially the visualization and quantitation components, and to produce a functional prototype. The prototype will be evaluated and validated against the standard and established laboratory based methods for gHb analysis.

B. Identification and Significance of the Problem

Diabetes mellitus is a chronic and potentially disabling disease which represents a major public health and clinical concern in the United States (1). People with the disease are at increased risk of developing chronic complications related to ophthalmic, renal, neurological, cerebrovascular, cardiovascular, and peripheral vascular disease (2). Diabetics are generally more likely than their nondiabetic peers to have heart attacks, strokes, amputations, kidney failure, and blindness. Diabetes mellitus is estimated to afflict more than 14 million people in the United States (3), with a total economic burden exceeding $40 billion per year. The Diabetes Control and Complications Trial (DCCT) (4) confirmed a direct relationship between the degree of blood glucose control and the risk of late renal, retinal, and neurological complications in patients with insulin-dependent diabetes (IDDM). Blood glucose control was confirmed by measurement of glycohemoglobin (gHb). gHb measurement plays a central role in the conclusions of the DCCT.

Glycated hemoglobins (gHb) arise from the non-enzymatic attachment of glucose, glucose-6-phosphate or fructose-1,6-diphosphate to hemoglobin. Of clinical interest are the glucose products which are formed and accumulate in the erythrocyte in proportion to the prevailing blood glucose concentration. Glycated hemoglobins have become an important parameter for the objective assessment of averaged long-term glycemia in patients with diabetes mellitus (5).

Measurement of glycated hemoglobins is especially useful in insulin-dependent diabetic patients where blood glucose concentrations fluctuate widely and where the fasting blood glucose does not reflect averaged glycemia, as is often the case in non-insulin-dependent patients. With regard to detecting glucose intolerance, glycated hemoglobin levels are less sensitive than results of oral glucose tolerance tests consistent with a conservative approach to the diagnosis of diabetes (6). An unaltered life span of the erythrocyte (7) and the absence of significant concentrations of the reactive galactose in blood (8) are prerequisites for glycated hemoglobins to yield reliable information concerning averaged long-term glycemia.

Human hemoglobin is quite heterogeneous because of mutation and posttranslation. More than 300 different kinds of mutant hemoglobins have been found. The replaced amino acid may bear a different charge to the original amino acid, so it may slightly affect the charge density of the whole molecule. Table 1 lists some of the many mutations; the names of these abnormal forms are derived from the location of their discovery.

Glycated hemoglobin is a nonenzyme-mediated, posttranslational modification, accumulating slowly in erythrocytes (Table 2). Human adult hemoglobin usually consists of Hb A (97% of the total), Hb A_2 (2.5%), and Hb F (0.5%) (9). Hb A consists of four polypeptide chains: two α-chains and two β-chains. Hemoglobin is glycated at several sites: the amino termini of both its α- and β-chains, as well as at certain ε-amino groups (10). Glycation of the amino terminus of the β-chains imparts enough change in charge to allow separation of the respective hemoglobins by charge-dependent techniques. Hemoglobins glycated at the termini of the β-chains elute from a cation-exchange resin in the HbA1 fraction well in front of the main hemoglobin peak. They are referred to in order of their elution as HbA1a, HbA1b, and HbA1c (Table 3).

Glucose is the carbohydrate in the major glycated hemoglobin, HbA1c, while glucose-6-phosphate and fructose-1,6-diphosphate are the two components of HbA1a. These phosphorylated carbohydrates are attached to the amino terminus of the β-chains only. HbA1b is also glycated, but its structure has not been fully established.

HbA1c is the major fraction of glycated hemoglobin,constituting approximately 80% of HbA1. The HbA1c level in blood depends on both the life span of the red cell (average 120 days) and the blood glucose concentration. The amount of HbA1c represents an integrated value for glucose over the preceeding 6-8 weeks and provides a criterion for assessing the diabetic state. Hemoglobins glycated at sites other than β-chain amino terminus elute in the leading edge of the main hemoglobin peak (11). This portion of glycated hemoglobin represents ca. 50% of total glycated hemoglobin and is detected by glycation-specific methods, such as the thiobarbituric acid (TBA) method or borate affinity chromatogrphy (12, 13). Almost all separations are based on the three methods noted in Table 4

It is widely accepted today that ion exchange (20-24) and affinity chromatography separation of diabetic hemoglobins provides the resolution necessary to meet the needs and recommendations of the DCCT panel. There is wide spread interest in providing gHb testing for all diabetics (17-20). It would be advantageous for tests to be available which could be performed on a point of care basis and even in the home environment A number of products are becoming available using ion exchange and/or immunoassay to help provide more simple means of testing and evaluation. These, however, are still designed to be used in a testing lab environment and are unlikely to be available for patient or point of care test use. Unfortunately, ion exchange and affinity chromatography generally do not lend themselves to effective testing outside of the clinical laboratory environment.

Given the very large number of Americans with type II Diabetes and the fact that half of these are undiagnosed and therefore at considerable risk to the chronic complications associated with this disease, it is imperative that screening and testing be much more widely availability (17-19). This is of particular urgency for the Hispanic and African American populations, as they have a higher prevalence of diabetes and suffer excess morbidity and mortality in comparison with the white population (19).

Table 1. Amino acid replacements in human hemoglobins (Ref. 16)

Chains	*Abnormal hemoglobin*	*Position*	*Normal residue*	*Replacement*
α-chain	I	16	Lys	Glu
	$G_{Honolulu}$	30	Glu	Gln
	Norfolk	57	Gly	Asp
	M_{Boston}	58	His	Tyr
	$G_{Philadelphia}$	68	Asn	Lys
	$O_{Indonesia}$	116	Glu	Lys
β-chain	C	6	Glu	Lys
	S	6	Glu	Val
	$G_{San\ Jose}$	7	Glu	Gly
	E	26	Glu	Lys
	$M_{Saskatoon}$	63	His	Tyr
	Zurich	63	His	Arg
	$M_{Milwaukee}$	67	Val	Glu
	D_{Punjab}	121	Glu	Gln

Table 2. Nomenclature and structure of relevant hemoglobins (based on Refs.16, 17)

Hemoglobin / glycohemoglobin	*Structure*
Hemoglobin	
A	$\alpha_2\beta_2$
F	$\alpha_2\gamma_2$
A2	$\alpha_2\delta_2$
*Glycated forms of Hb A (glycohemoglobin)**	
A1a1	α_2 (β-N-FDP)$_2$
A1a2	α_2 (β-N-G6P)$_2$
A1b	α_2 (β-N-CHO)$_2$
A1c (labile)	α_2 (β-N=Glc)$_2$
A1c (stable)	α_2 (β-N-Glc)$_2$
	α_2 (β-LysN-Glc)$_2$
A-Glc	(α-LysN-Glc)$_2\beta_2$
	(α-N-Glc)$_2\beta_2$

*FDP=fructose-1,6-diphosphate; G6P=glucose-6-phosphate; Glc=glucose; CHO=unidentified carbohydrate.

Table 3. The percentage of gHb and nonglycated Hb in normal and diabetic patients (Ref. 5)

nonglycated Hb / gHb	*Percentage of total Hb**	
	Normal	*Diabetic*
Nonglycated Hb		
A	97	80 to 90
F	0.5	0.5
A2	2.5	2.5
gHb		
A1a1	0.19 ± 0.02	0.20 ± 0.03
A1a2	0.19 ± 0.4	0.22 ± 0.04
A1b	0.48 ± 0.15	0.67 ± 0.3
A1c	3.3 ± 0.3	7.5 ± 2.0

*Mean ± 1 standard deviation

Table 4. Methods for estimation of glycated hemoglobins (based on Refs. 15, 17)

Measurements	*Methods*	*Proteins measured*
Charge differences	1. Cation-exchange chromatography	HbA1c
	2. Electrophoresis (agar gel, cellulose acetate, isoelectric focusing)	
Affinity	1. m-Aminophenyl boronic acid affinity chromatography	Total gHb*
	2. Immunoassay (radio, enzyme)	Specific gHb
Chemical reaction	1. Hydroxymethylfurfural -thiobarbituric acid colorimetry	Total gHb
	2. Periodate oxidation method	

*gHb = glycated hemoglobin

C. Background and Experience

Protein Solutions, Inc. is establishing itself as The Personal Chemistry Company. We are developing a range of sensors and devices with which to empower patients and consumers with the resources, motivation, and skills to monitor their own personal chemistries and to aid their health care providers in the detection and management of disease. Our work to date has focused on the development of direct reading sensors for the management of chronic metabolic diseases, in particular PKU and galactosemia. We expect to extend and expand that work to the monitoring of homocysteine and critical vitamins.

Much of our technology and expertise is directly applicable to the separation of proteins for the analysis and monitoring of a variety of protein dependent diseases and states We have considerable experience in protein separation and detection. Our skills in low molecular weight metabolite analysis and our skills in protein separation and detection will lead to far more efficient and effective devices and instruments with which to monitor and manage both Type I and II diabetes.

Protein Solutions, Inc. has had a close and synergistic relationship with the University of Utah. Joe Andrade, President and Chief Executive Officer, is on the faculty in the Departments of Bioengineering, Materials Science and Engineering, and Pharmaceutics. He and his group have a long track record of work in protein adsorption and related interfacial properties (30-36). Already many years ago, this fundamental work utilized gradient methods to more effectively and efficiently evaluate protein surface interactions. These gradient methods were, in principle, not unlike the gradient elution techniques used in conventional ion exchange and hydrophobic chromatographies and the immobilized pH gradient methods used in isoelectric focusing. Although such methods have proven to be very effective in the high resolution separation of proteins, they have rarely been used outside of a research or clinical laboratory environment.

Four years ago Protein Solutions, Inc. began to develop simple direct reading biosensors for low molecular weight metabolites of clinical interest, based on the unique specificity and sensitivity of the ATP dependent firefly luciferase and NADH dependent bacterial luciferase bioluminescence reactions. The development of such sensors, and particularly the means to make them rigorously quantitative, with very simple luminometric instruments or even by direct eye visualization, required the development and application of some unique enzyme gradient technologies (29-31).

Joe's group had felt for a long time that it should be possible to incorporate the highly effective enzyme/protein gradients used by PSI's bioluminescent sensors, the surface property gradients used in their basic studies of protein adsorption, and the gradient approaches used in high resolution protein chromatography and electrophoresis. We agree. We feel that, in principle, these various technologies will form the basis of "simple" planar, perhaps dipstick-like, devices for the high resolution separation of proteins of clinical and diagnostic interest.

About three years ago, Joe and a particularly creative and hard working Ph.D. student, Q.L. Luo, began to tackle the problem. In the past three years, Mr. Luo has made remarkable progress in developing planar, thin layer-based, protein separation devices using the principles of ion exchange chromatography (33-36).

A thin layer chromatography (TLC) device is not necessarily a simple device. The devices we envision are actually quite sophisticated, although their final application by the ultimate user is very simple.

Dipstick devices generally have a bad name in clinical chemistry and medical circles because they are normally based on colorimetric technologies and color matching processes, whose reading depends on the observer's color perception. Such devices are difficult to calibrate and standardize. More recently, the development of linear reading devices, more or less "thermometer-like," such as the immunochromatographic (25) drug assays and the over the counter cholesterol test, have started to improve the reputation of dipstick analytical devices. The quantitation of color based clinical chemistry reactions has also been dramatically improved with the development of small, inexpensive, hand held quantitative colorimeters, best represented today by the wide range of glycometers available in any retail drugstore. The analytical device is still a dipstick, but it is measured by an instrument appropriately designed to minimize the problems associated with quantitative colorimetry.

The advantages of a planar TLC-like device are many: ease of sample application, capillarity driven sample or fluid flow, planar geometry and design, the use of dry reagent technologies to minimize liquid handling and dilution steps, and the potential for direct reading and quantitative visualization.

The problems with developing a thin layer or dipstick-type high resolution protein separation device, however, are substantial. Luo and Andrade have considered hydroxyapatite as a thin layer sorbent and have extensively worked with more conventional ion exchange media. They have also worked extensively with pH and ionic strength based mechanisms, as well as with displacement chromatography (32-36). They have developed a unique step gradient technique which optimizes, but greatly simplifies, the highly effective gradient elution methods used in high performance column chromatography.

We propose to develop "simple" dipstick-type device for the separation and analysis of glycated hemoglobin. That is the major objective of this Phase I application.

Separation of the hemoglobins is problem 1 and the key objective of this Phase I application. Problem 2 is the visualization of those separated bands, preferably by non colorimetric means. Problem 3 is the quantitation of those bands. The goal is a device which permits the detection and quantitation of those various hemoglobins important in the management of chronic Type II diabetes.

Problem 1 will be discussed in the next section. Here we briefly discuss Problems 2 and 3, the key parts of Phase II.

Fortunately, hemoglobin is directly visible due to the presence of the heme chromophores. Analysis utilizing a relatively large volume of blood involves sufficient hemoglobin, including the glycosylated variants, that the separated bands can be directly visualized. Our goal, however, is to utilize the minimum blood sample feasible, of the order of 100 microliters or less, obtained generally from a very light, lancet, fingertip puncture. This will require enhanced visualization methods, particularly for the relatively low concentration glycated fractions. We are assuming that as more is learned about the glycosylation of hemoglobin and of the plasma proteins there will be growing interest in the separation and detection of those glycosylated variants which are at present largely ignored. This will require an enhanced sensitivity detection system.

In addition, as noted earlier, colorimetric-based analyses are difficult to quantitate by direct visual means. We will now discuss our approach to the high sensitivity detection and quantitation of separated proteins.

There are two very special molecules that play unique and central roles in biology: adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) and its phosphate form (NADPH). ATP is generally recognized as the energy currency in biology. ATP and NAD(P)H are closely coupled in many biochemical processes and can be regenerated or recharged. They are the basic coupling agents of cellular metabolism. A very large number of biochemical enzyme processes involve one of these two molecules.

It is very fortuitous that biology has evolved two bioluminescent processes dependent on these two molecules: the firefly luciferase reaction, which acts on firefly luciferin in the presence of ATP to produce an oxidized product which chemiluminesces. The bacterial luciferase reaction, which in the presence of alkyl aldehydes and $FMNH_2$, produced by an NAD(P)H reaction, also produces an excited chemiluminescent product which chemiluminesces. Both reactions produce photons with high efficiencies in the presence of oxygen. However, both the luciferases and luciferins involved are chemically different.

There is a large body of literature on the development of biosensors for ATP and ATP-dependent processes and for NADPH and NADPH-dependent processes, using the firefly and bacterial luciferase enzymes, respectively (29). Such biosensors generally employ fiberoptic or other wave guided means of delivering the luminescence to a device which can accurately measure light intensities. Although one of the most portable and most sensitive photon detectors available to the scientist, physician, or patient is his or her own eye, it is notoriously difficult to calibrate for accurate measurements of even relative light intensity. The human two dimensional photon detection system, however, can reliably and accurately measure changes in spatial position.

We have developed a set of technologies which allows ATP concentration to be measured by the spatial position of the bioluminescence, permitting a quantitative detector designed and optimized for human visual detection. The "trick" is to display the signal in space. The general concept is presented in Figure 1 (30, 31).

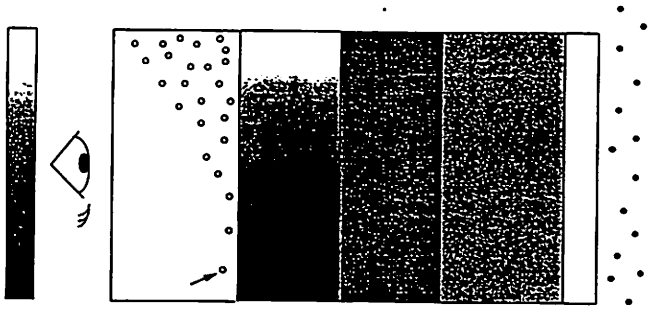


Figure 1. The "Business Card" geometry dipstick sensor, read by looking at the far right edge. Liquid sample is applied to the far left edge (See the text for details).

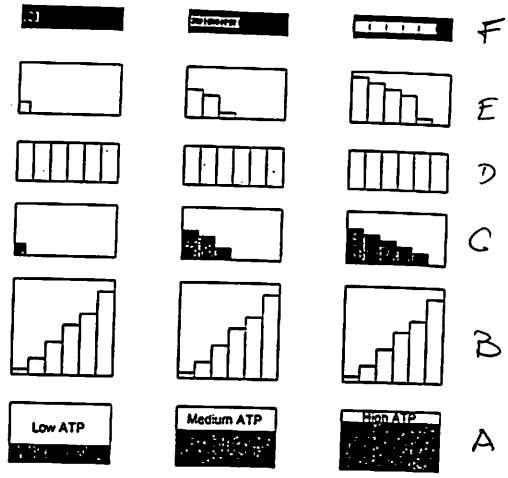


Figure 2. (right) The mechanism of action of the sensor. The bottom blocks (A) refer to concentration of ATP in the sample. The next block up (B) is the apyrase distribution; the center blocks (C) are the resultant ATP concentrations. D refers to the luciferase/luciferin zone. E is the light output, and F is what you see looking at the upper edge of the sensor

Imagine the sensor with the shape of a thick business card. One dips the left end of the business card (Figure 1) in the solution to be analyzed. The liquid sample wets and is wicked into the card. Let us assume for now, for simplicity, that we are only interested in analyzing the ATP content of that sample. We are describing the generic ATP detection sensor. Assume that the solution has a uniform ATP concentration. It moves into a region where reagent for pH and buffer control has been deposited. The dry reagent is solubilized and the now buffered pH controlled ATP containing solution moves into a zone which we call the "consumase" gradient. Here we have an enzyme which consumes ATP. You will see why in just a moment. The gradient means that ATP is consumed in a position sensitive manner – high consumase concentration at the top of the figure, and a zero consumase concentration at the bottom. The spatially modulated ATP now makes its way into the final zone of the sensor which contains luciferase and luciferin, the two reagents which together with oxygen and ATP produce the bioluminescence. At the top of the device, where the consumase was highest, there is very little ATP. At the bottom of the device where it was lowest, there is a maximal amount of ATP. Now imagine that you are observing light generated by the bioluminescent process by looking at the edge of the business card. What you see is a very thin band, very dark on your right (top) and relatively bright on your left (bottom). It is clear that the position of the light in that band has some relationship to ATP concentration. Now to Figure 2.

Here we have it in a more schematic version. At the far left imagine a sample containing low ATP. That sample enters a zone in which there are six discrete consumase concentrations. You can think of this as six independent channels if you like, each accepting an identical volume of solution of identical but low ATP concentration. In this particular example, the consumase is of high concentration and the ATP concentration is low so that essentially all the ATP is consumed except in the left most channel. A small amount of ATP thus enters the next zone, the luciferase zone, and results in light but of relatively low intensity. At the far right of the figure we have a sample of high ATP concentration. Now the ATP concentration is high enough that even though much of it is consumed by the consumase, a sufficient amount gets through into the luciferase zone, producing light. There is more ATP on the left side of that high ATP example and so the light is of course brighter. Think of the output of these sensors, i.e. the top of Figure 2, as analogous to a glowing thermometer. The length of the glow is thus proportional to ATP concentration. The intensity of the glow is also related to ATP concentration (it is the intensity that is normally measured but requires a relatively expensive intensity detector). In our case, as long as the glow is above the detection threshold for the human eye, and the non-glowing region is black

enough that the human eye can detect significant contrast between the glowing and non-glowing region, then that particular position is indicative of ATP concentration.

This works remarkably well, as shown in Figure 3. This is a laboratory example in which the consumase (potato apyrase) has been deposited in one dimension of an eight by eight multi-well plate and ATP solutions ranging from 10^{-7} to 10^{-4} molar have been placed in the other dimension. The wells already contain luciferase and luciferin of constant concentration. Examining this plate after one minute of equilibration and reaction time, one sees that indeed the position of the glow is proportional to ATP concentration. Note that in the case of very high ATP concentration, the glow/no-glow region is at position A, whereas at 10^{-5} it is approximately at position C, and at 10^{-6} it is position E. Visual detection is quite straight forward. This particular image was taken with a CCD camera. Photographic recording is also very rapid and sensitive. This particular example was designed to have a wide dynamic range, three orders of magnitude in ATP. One can design the gradient for a one order of magnitude range with about ten percent accuracy within that range. So, depending on the design of the apyrase steps, one can produce a sensor with any requisite sensitivity and range.

This is what we refer to as the generic ATP sensor, or the ATP detection platform. This is all background and experience.

We can use this generic ATP detection platform as a basis for a range of sensors for carbohydrates, amino acids, vitamins and other metabolites, but that is not the subject of this application. We propose to use this ATP detection platform as the means for quantitating the protein bands separated by the thin layer, planar, chromatographic device.

The preliminary design of the device is given in Figure 4 (next page). This figure is organized vertically so that the reviewer can make a photocopy of the page, cut out the figure, and fold and manipulate it as indicated in the caption. This way the reviewer will have much better appreciation and understanding of what is being proposed. The extensive two page caption describes the device and its operation (to next page please).

Admittedly, Figure 4 seems a bit complicated It is, indeed, quite complicated, but will be designed, engineered, and implemented in such a way that most of the complication is "transparent" to the user, just as the highly sophisticated computer programs we use today are relatively transparent and easy to use, and just as your microcircuit wrist-mounted time piece is very highly sophisticated from a micro and nano engineering and fabrication point of view, but it is exquisitely easy to apply and read.

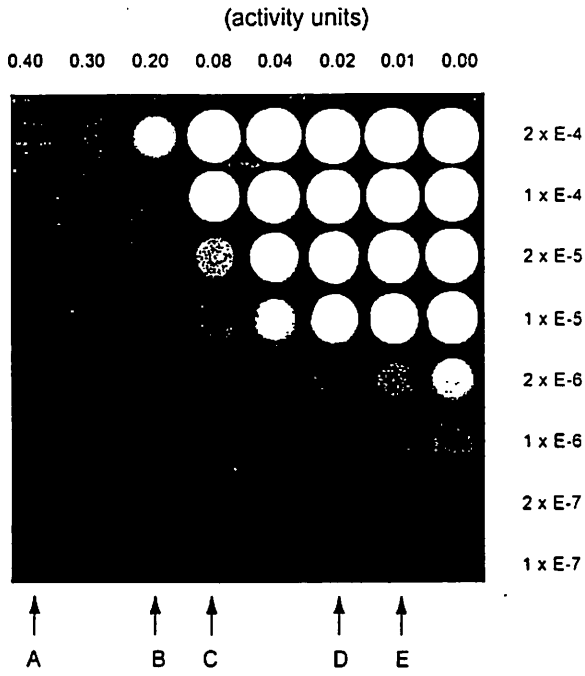


Figure 3. Eight ATP sensors (horizontal) each with identical consumase step gradients. Solutions of various ATP concentrations (right, vertical axis) have been applied to each of the sensors (see text for details).

The device will simply require applying blood as is done now for quantitative glucose testing in the home environment, and applying a pre-measured elution buffer to provide sufficient solution volume to facilitate the rest of the process. There will be a simple visual indicator as to when the separation has proceeded sufficiently for the first blotting or clamping phase, followed by a second visual indicator to note when that first phase should be unclamped and the second blotting

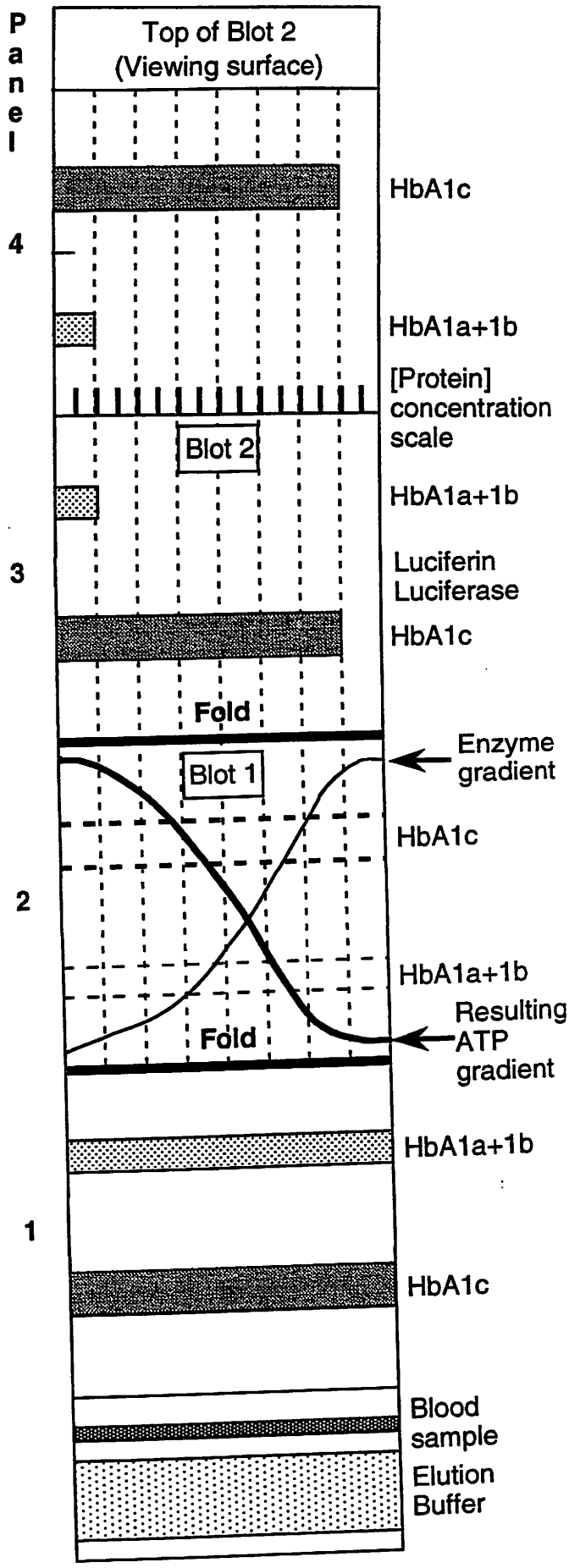
Figure 4. A very preliminary design for a dipstick device appropriate for the high resolution separation, visualization, and quantitation of hemoglobins appropriate to the monitoring of chronic diabetes. The device is shown at the right as a long thin card designed for folding on the horizontal dotted lines as indicated. The bottom portion of the card is the sample application, red cell hemolysis, hemoglobin lysate transport and separation zone. The blood sample is applied at the bottom of the card by the application of two or three small drops of blood obtained from an ear lobe or fingertip lancet procedure as is routinely done for the monitoring of glucose in the home environment. The blood sample region contains a hemolytic agent which lyses the cells and releases the Hb. A larger volume transport and elution buffer is then applied to the larger zone immediately below the blood sample zone. The solution will be provided in a small disposable pipette and provides the only wet chemistry step necessary for the application of this device.

The remainder of the bottom panel of the device is the separation region. The elution buffer migrates along the plate, carrying with it the hemoglobin lysate. Plasma proteins have already penetrated down deep into the paper where they are largely immobilized and will not migrate and contribute to the protein separation bands. The device, therefore, intentionally eliminates plasma proteins and separates only the red blood cell lysate.

The key to the separation is to have dry reagent bands pre-impregnated in the separation zone to facilitate the separation of the various hemoglobins. These bands may include various pH buffer zones and ionic strength zones. In addition, the sorbent itself may be designed with a step gradient including both ion exchange and affinity components.

The elution buffer has appropriate volume to facilitate optimum separation of the proteins. Excess buffer is taken up at the top of the panel if needed, as indicated. Optimum separation is the major objective of this application. Let us now assume that seperation has been successful and of sufficient resolution and precision to be appropriate to the measurement of glycosylated hemoglobins.

The next step is the visualization of these protein bands. They are no longer moving because the excess elution buffer has been absorbed at the top layer of the separation zone. Now we configure the device so that the Blot 1 region can be folded against the separation region, that is Panel 2 is contacted with Panel 1. This would, of course, be designed in an appropriate plastic container with a built in clamp so that it could be snapped in place and held uniform pressure.



The purpose of this blotting region is to generate a signal which is proportional to the amount of protein present and to the position of each protein band.
This is where our unique bioluminescence gradient detection technology comes in. Assume that Panel 2, the Blot 1 zone, has a uniform concentration of ATP. Further assume that there is a horizontal gradient of an ATP dependent enzyme placed across that zone as indicated in the drawing. The enzyme catalyzes an ATP dependent reaction of the protein in each of the separated bands. We assume that the protein band is uniform along the horizontal dimension, which is also the dimension of the gradient. As the enzyme makes contact with the hemoglobin band and degrades and/or phosphorylates it, the local ATP concentration will be diminished. Where the enzyme concentration is high, the ATP will be significantly depleted; where the enzyme concentration is low, there will be essentially no depletion of ATP. There is sufficient moisture in Panel 1 to fully activate the dry deposited enzyme and to solubilize the low molecular weight ATP. The reaction is fast enough, however, that the ATP does not have sufficient time to diffuse and thereby blur or invalidate the gradient. It is possible that the gradient will not be continuous as indicated in Panel 2, but rather will be step wise which is indicated by the light vertical lines in Panels 2 and 3.

Assume this blotting process has occurred. The enzyme has acted on the separated hemoglobin. The ATP has been consumed, and we now have an ATP gradient as shown. We now take the upper Panel 3 of the device, called Blot 2, and fold it in to make contact with Blot 1. So, Panels 1 and 2 are now unconnected and Panels 2 and 3 are now making contact. Panel 3 contains a thin dehydrated film of firefly luciferase and luciferin in a pH buffered environment optimal to the activity of the luciferase. We now simply contact Panel 3, which is also called Blot 2, against Panel 2, or Blot 1. They snap in position. The excess buffer which was absorbed in Panel 1 and is now in Panel 2, also gets transferred to Panel 3 and very rapidly hydrates that panel during the initial contact and clamping process.

Now assume that Panel 3 is appropriately hydrated, the luciferase is activated, the luciferin is solubilized, and it makes contact with the ATP gradient in Panel 2. Gradient-based bioluminescence activity across the original protein separated band is what gives us the quantitation, not unlike what you saw earlier in Figures 1-3, showing our ability to detect ATP concentration by spatial position.

Now look at Panel 4. Panel 4 and Panel 3 are front and backs of the upper section of the device. Panel 4 is the back side of Panel 3. That is difficult to show in a proposal where you have to photocopy on one side only, but if you simply fold Panel 4 over on the backside of Panel 3 and look at it, you will see how the sensor is visualized. The bioluminescence intensity resulting from the Panel 2/3 contact is actually observed through the backside of Panel 3. Let us now assume that it is the top surface (Panel 4) that you are looking at. Each of the originally separated protein bands is pre-labeled on that top surface with the appropriate identification. This is, of course, all worked out in the calibration and validation parts of the Phase I and Phase II projects. Thus, the separation grid is super-imposed on Panel 4. The ATP gradient, however, has lead to a bioluminescence gradient along each individual protein band. Again, this is analogous to the spatial positioning of bioluminescence, the key part of our direct reading sensors.

Now, one simply reads the glowing "thermometer" of bioluminescence for each hemoglobin band. The band is detected, the length of the glow is proportional to the amount of protein present, which is a result of the enzyme specific ATP depletion reaction which occurred. The operator can now simply mark the end of the glow and read that off against a pre-labeled concentration index. Indeed, it will be possible to remove that portion of the sensor and to literally file away that part of the device as a document in the patient's record.

Yes, there are a number of questions regarding the quantitative visualization process. For example, the intensity appropriate to direct visualization? These are issues that we have addressed in our current sensor development efforts. They will be appropriately considered in the Phase II application. Briefly, the device will be viewed in a simple black cardboard viewer, which will facilitate minimizing the influence of ambient light. The reader or operator will not need to be dark adapted and will not have to assess the intensity of the light, but rather only its position. End caption.

or clamping phase applied. A final visual indicator will note when the operator should mark the device for permanent recording and evaluation.

Admittedly, no one has, to our knowledge, made such a multi dimensional dipstick-type quantitative direct reading protein analytical device. We feel that we have the experience, technology, and motivation to do so.

Our discussion of the bioluminescence-based quantitaion portion of the device has assumed an ATP dependent protease. We are in the process of acquiring and studying such enzymes (28). This is a key part of a parallel effort in the development of protein specific sensors. In the event that this approach does not prove practical, there are a number of alternative means appropriate to the detection of hemoglobin. We will assess the practicality of using a heme oxygenase, which oxidizes heme to bilibrubin and requires NAD(P)H. The NAD(P)H can, of course, be assessed by bacterial luciferase bioluminescence reaction. A description of such a detection system is directly analogous to that already given in Figure 4 for the ATP dependent process, except that we would use the heme oxygenase, which by virtue of its action on the hemoglobin, consumes NAD(P)H, leading to its gradient. It would then be detected by the upper panel using an NAD(P)H sensitive bacterial luciferase. We are already utilizing the bacterial luciferase system for sensors for lactate and phenylalanine and have considerable experience with quantitative gradient approaches using bacterial luciferases (37).

Experimental Design and Methods

1. Evaluate means of separating and lysing red blood cells, permitting hemoglobin to be released and transported to the front end ion exchange portion of the device.

The blood application zone at the very bottom of Figure 4 and Panel 1 would be designed so that the plasma permeates down into the pad where the plasma proteins are immobilized and prevented from eluting with the hemoglobin. The red cell fraction remains near the surface of the pad. That region contains a partially immobilized cocktail of surfactants and related agents which hemolyse the red cells. At the appropriate time, the elution buffer is added to the zone below the blood sample (see Figure 4). As the elution buffer is wicked into the upper portion of the device, the released hemoglobin is carried with it. The surfactant cocktail and the rest of the blood containing region are constructed to minimize the migration of surfactants, membrane fragments and other constituents, allowing a relatively pure hemoglobin lysate to migrate into the separation zones of the device.

Generally, such processes are accomplished in a clinical lab by a variety of steps often including multiple centrifugation steps to assure a clean hemoglobin preparation. We, of course, do not have the luxury of multiple steps and centrifugation here. Rather, the preparation of a relatively pure hemoglobin lysate must all be accomplished by dry reagent technologies. We will evaluate a range of commercially available absorbent pads for the separation of plasma from red blood cells and a range of surfactants, including macromolecular surfactants, to facilitate surfactant immobilization.

2. Develop a thin layer cationic exchange device for the capillarity driven, pH ionic strength gradient modulated, separation of hemoglobins. This task will involve the evaluation of a number of commercial cationic exchangers and the determination of the optimum pH gradient and ionic strength gradient conditions for maximal separation and resolution.

The development of the optimized cationic exchange thin layer separation system, the upper two thirds of Panel 1, Figure 4, can be accomplished with an ultra simplified version of the dipstick device, indicated in schematic form in Figure 5. Coated dipstick refers to an appropriate support on which the cationic exchange resin has been deposited in a thin layer format. This work is quite well advanced for plasma protein separation (32-36). The initial goal is to duplicate the relatively high resolution ion exchange separation now used for gHb analysis.

gHb is separated on a weak acidic cation-exchanger consisting of carbonyl ($R\text{-}COO^-$) groups attached to a polymer lattice. At a neutral pH, the Hb components are positively charged. The minor components are less charged than HbA_0 and do not interact as well with the stationary phase. By gradually increasing the concentration of cation, usually sodium, in the mobile phase, the components are eluted from the stationary phase in order of increasing positive charge. The minor hemoglobin components flow through more quickly than HbA_0 providing excellent separations under proper assay conditions.

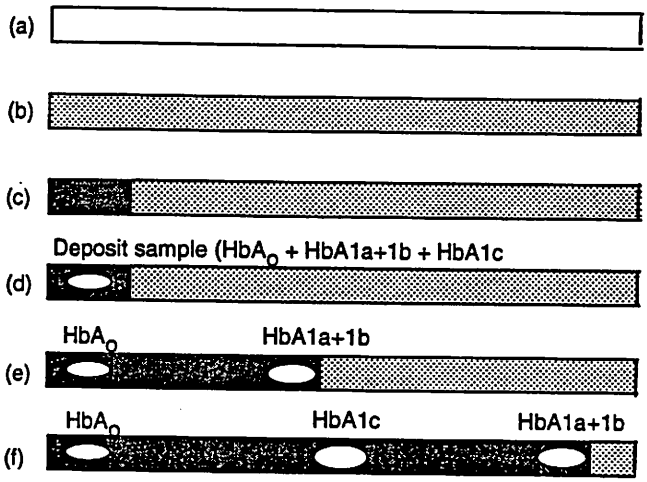


Fig. 5 The illustration of step elution process in cation-exchange chromatography. (a). Uncoated dipstick; (b). coated dipstick; (c) wetting for depositing sample; (d) deposit sample; (e). first developer is used; and (f). second developer is used.

Cation-exchange chromatography is the most widely used separation method for gHb analysis and is the standard. Both microcolumn chromatography and high performance liquid chromatography (HPLC) are commonly used. Microcolumn uses a two-developer system: the HbA1a+1b fraction is eluted first using a developer containing a very low sodium ion concentration followed by elution of HbA1c using a second developer. HPLC uses a similar elution method to that used in microcolumn chromatography, but shows enhanced assay precision and permits rapid separation of HbA1c from both the other minor hemoglobin components and the major hemoglobin fraction. Chromatograms from diabetic and nondiabetic samples show that the HbAlc and HbAla+b fractions are adequately resolved (5) (Figure 6). More complete separations of the minor hemoglobin fractions require slower elutions and do not significantly improve assay precision and operation. Glycated and non glycated hemoglobin components can also be separated by electrophoresis or isoelectric focusing. Initial set-up costs are similar to those for HPLC. These methods has not found widespread use in clinical laboratories due to equipment and operating cost issues. Several chemical approaches have been used to measure glycated proteins (Table 4). These generally focus on the carbohydrate moiety.

The high resolution separation ideally requires an ionic strength gradient, shown in Figure 6, a standard HPLC analysis. The trick is to incorporate that gradient in a device, as indicated in Figures 5 or 4, without having to resort to second or subsequent elution steps. This is done by pre-depositing the components of the gradient directly on the dipstick so that the gradient evolves during the capillarity driven elution process. This is the "trick" which permits high resolution separation in a "simple" device. We will study this process using pH gradients which can be directly and readily visualized using colorimetric pH indicators. This will very quickly allow us to study buffer deposition and control the eluent wicking rate and volume, to generate the appropriate buffer compositions and consequent pHs. We will then apply the principles and experience learned to the preparation and development of ionic strength gradients to essentially duplicate the separation of hemoglobin, indicated in Figure 6. This particular task or specific aim is really the major part of this Phase I proposal. We will evaluate a range of cationic exchange resins, papers, and other matrices.

3. Evaluate and optimize an affinity chromatography second stage to more fully and completely separate the gHb bands. The affinity chromatography second stage will also assess the feasibility of a boronic acid affinity technique.

Cross-linked agarose (matrix) coupled to *m*-aminophenyl boronic acid (ligand) has been shown to provide an efficient matrix for separating and quantitating gHb (Table 4); since the cis-diol groups in glucose-modified hemoglobin are co-planar, reversible five-membered ring complexes are formed with the borate ligand. Unbound glucose has the appropriate conformation to interact with the ligand, but studies indicate that it does not interfere unless present in very high concentration (greater than 500 mg/dl). The glycated Hb components are retained by the column. The bound fraction is then eluted with sorbitol, a counter-ligand which competes with the bound glycated materiel for the boronic acid binding sites. The specificity of affinity chromatography differs considerably from methods that separate the various forms of gHb based on their charge differences. Even by HPLC, the HbA1c fraction contains both glycated and some nonglycated hemoglobin (e.g. HbF); similarly, the HbA_o peak contains both some glycated material. However, by affinity chromatography the separation is more specific for glycation. The bound fraction contains both HbA1c and an appreciable quantity of HbA_o, glycated at several sites other than the N-terminus of the β-chain.

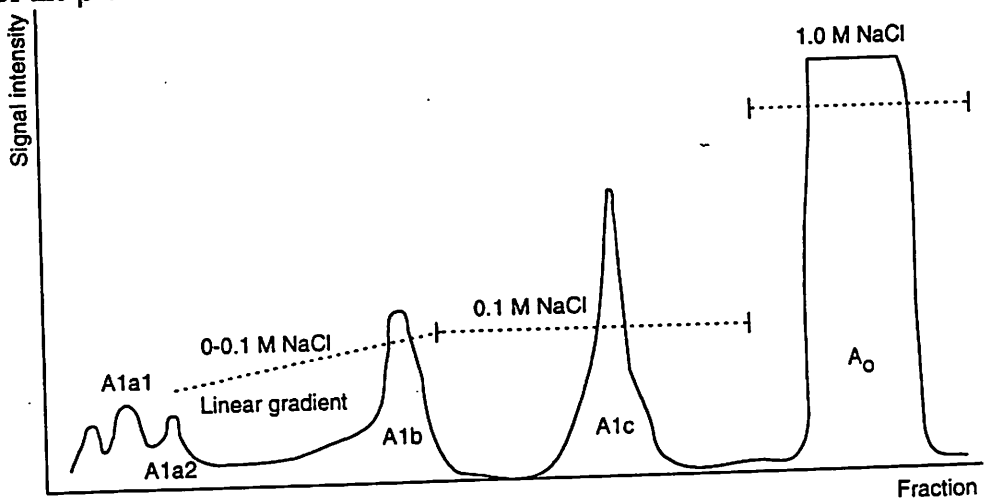


Fig. 6. Elution profile of normal human hemolysate chromatographed on Bio-Rex 70 cation exchange resin. Hemoglobins A1a1, A1a2, A1b, and A1c are posttranslational modifications of Hb A(A_o) ref.5).

Measurement of gHb using boronic acid affinity chromatography is a useful alternative to other gHb assay methods. The assay is fast, inexpensive, and less affected by small variations in temperature or pH than other commonly used methods. In addition, the technique shows substantially less interference from certain factors, such as aldimine intermediates, hemoglobinopathies, and storage-related alterations. However, this method is still under development.

It may be possible to improve or enhance the separation using an appropriate affinity technique, however. By incorporating an affinity stage in the sensor, we may able to simplify the ion exchange component of the device, perhaps eliminating the need for the gradients described above in Specific Aim 2. For this reason we will study the application of boronic acid affinity chromatography, as this has been shown to be a highly effective tool for the separation of glycated proteins.

Recently, hydrophilic vinyl based borate matrices have been used as affinity supports. These can be easily coated on plates in a thin layer format as indicated in Figure 7. Boronate

affinity chromatography for gHb requires a carefully optimized buffer. We will begin with 100 millimolar taurine at pH 8.5. The eluting buffer is generally 0.1 molar sorbital and taurine.

4. Begin the integration of the four basic components of the device: red cell separation and lysis, hemoglobin transport and delivery, ion exchange separation, and affinity separation. Further optimization of separation and resolution will involve optimization of device dimensions, volumes, and separation times.

The result of Specific Aims 1 to 3 will be combined and optimized in the final two months of Phase I to develop an integrated device for gHb separation. We expect to have a pre-prototype, which will be a hybrid of Panel 1 of Figure 4 and Figures 5 and 6. Basically it will be a dipstick device, releasing and separating hemoglobin from applied blood samples, using an optimized combination of cationic exchange and borate affinity chromatography in a TLC format. The only visualization we will have available at this stage is the absorbance, the color, of hemoglobin itself. This will be sufficient for these Phase I studies.

There are other means to enhance the separation and the visualization. These involve careful consideration of sample volumes, elution buffer conditions and volumes, migration and elution rates, and the dimensions of the device itself. These variables are all reasonably well understood in the dry reagent/dipstick technology field.

The basic aim here is to produce a pre-prototype TLC separation device. Most of the optimization and enhancement of this pre-prototype will be the subject of the Phase II application and work.

5. Evaluate an ATP-based labeling system to permit quantitative detection using the highly sensitive and specific firefly luciferase technique. Most of this work will be done in Phase II.

During the course of the Phase I project, we will be carefully considering the means for high sensitivity visualization and quantitation of the separated hemoglobins. We will continue our assessment of an ATP dependent protease approach as noted earlier (28), the heme oxygenase approach, utilizing an NADPH dependent reaction (26, 27), and perhaps others. The specific approach for visualization and quantitation using our spatial gradient method for direct visual quantitation will be detailed in the Phase II application.

Phase III: Corporate partners for Phase III are now being contacted. We will first approach those firms with a strong interest in and market commitment to devices and methods for the diagnosis and monitoring of diabetes. The technologies required for the analysis of glycohemoglobin are substantially different from those for the measurement of glucose and related metabolites. In order for a firm to have a complete product line for diabetes, those firms presently selling glucose dipsticks and glycometers will need to be able to supply devices for the measurement and monitoring of glycated hemoglobins. Ideally, the various devices will be coordinated and made available in a multi-panel device so that a single blood sample will provide multiple channels of diagnostic and monitoring information.

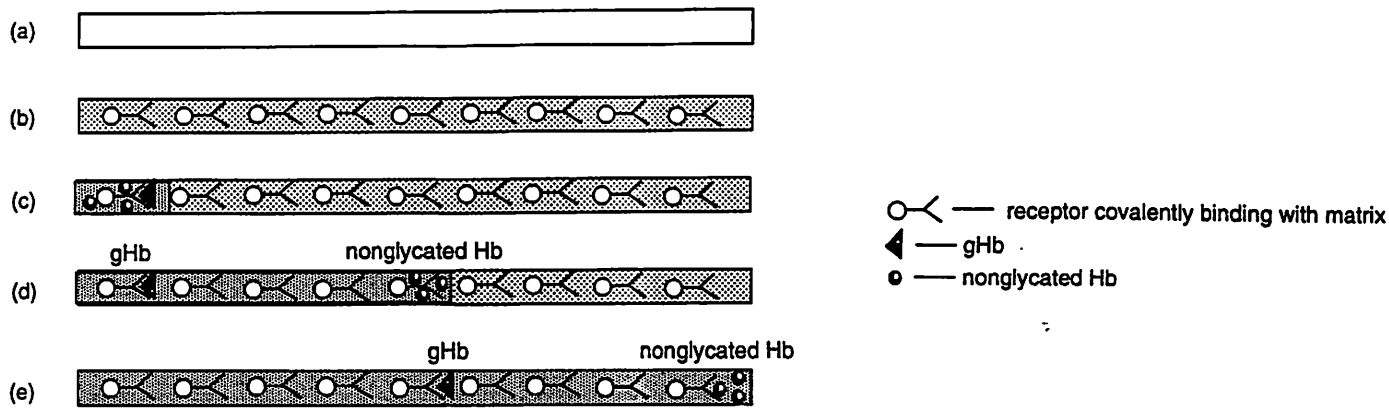


Fig. 7. The illustration of elution process in affinity chromatography. (a). Uncoated dipstick; (b). coated dipstick; (c). deposit sample; (d). washing; and (e). elution.

E. Human Subjects (none)

F. Vertebrate Animals (none)

G. Advisors

No paid consultants are budgeted. Three key advisors are Dr. Larry Kricka, Dr. Russell Stewart, and Dr. Joseph Andrade. Larry and Russell serve on the Scientific Advisory Board and are paid consultants on our on going Phase II STTR National Science Foundation grant on ATP-based biosensors.

Dr. Larry Kricka is Director of the General Chemistry Laboratory and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania, Philadelphia. Dr. Kricka is internationally recognized for his work on applying bio- and chemi- luminescence to clinical chemistry. He is editor of the *Journal of Bioluminescence and Chemiluminescence* and editor/author of many books on Bio- and Chemi-luminescence in clinical biochemistry. We interact with Dr. Kricka several times each year. He will provide appropriate advice and guidance to this project as part of his service on the Scientific Advisory Board.

Dr. Russell Stewart is Assistant Professor of Bioengineering at the University of Utah and PI of our University of Utah STTR Phase II subcontract on recombinant firefly and bacterial luciferase. He will advise and assist in areas of protein engineering, production, and characterization as needed.

Dr. Joseph Andrade is founder, President, and CEO of Protein Solutions, Inc. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. Five years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. Joe will be available to assist and consult in the areas of interfacial biochemistry, bioluminescence, and biosensor expertise when required.

H. Contractual Agreements (none)