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A Proposal to: Date: 8/15/02

Fresenius Medical Care
Renal Product Technologies
475 West 13th Street
Ogden, Utah 84404

From:
Principle Investigator: University of Utah, Dept. of Bioengineering
Joseph D. Andrade
Lab for the Modeling, Measurement, and Management of the Metabolome (4M Lab)
University of Utah
50 S. Campus Center Dr., Rm. 2480 MEB,
Salt Lake City, UT 84112-9202
Phone: 801-581-4379 Fax: 801-585-5361
joe.andrade@m.cc.utah.edu
www.bioen.utah.edu/faculty/jda

Title: **Bioluminescent Assays for Urea and Phosphate: Towards a DialysisChip**

Abstract:

Chronic renal failure patients should be regularly monitored to assure adequacy of dialysis and nutritional status. The bioluminescent assay for urea, which is currently under development in aqueous form, will be modified for dry reagent (dipstick) application. The urea assay will be lyophilized for eventual application in a DialysisSensor or RenalSensor. The dry reagents will be reconstituted with the analytical sample (blood, dialysate, urine). The bioluminescent assay for phosphate will be developed as an aqueous assay with experiments including optimization of buffer type, pH, reagent concentration, enzyme ratio, enzyme concentration, and other appropriate parameters. The optimized aqueous assay will serve as a starting point for lyophilized or dry reagent development. The development of the lyophilized or reconstituted assays will consider storage time and conditions, additive/stabilizer type and concentration, and reconstitution time and conditions. The assays will be compared with and validated against existing standard methods.

Background and Significance:

The development of a cost-efficient, home-care, low-sample volume biosensor for urea and phosphate would be useful to peritoneal dialysis and hemodialysis patients as well as to the development of more efficient dialyser cassettes and associated materials. A biosensor for measurement of urea in blood would be particularly important for home peritoneal dialysis patients so that the physicians would have serum concentration data without the need for a costly and time-consuming patient visit [1, 2]. Peritoneal dialysis patients currently compose approximately 15-17% of renal dialysis population. The current push towards more home hemodialysis means a biosensor would offer a needed and convenient service to hemodialysis patients and their physicians. Adding additional chemistry sensing channels to future versions of the biosensor is straightforward.

In general, dialysis patients absorb more phosphate from their diet than can be removed with treatment; therefore, phosphate levels are reduced by either reducing dietary intake or the administration of phosphate binders. However, due to lack of patient compliance and side effects from phosphate binders, increased frequency of hemodialysis may be necessary to reduce phosphate levels[3]. Maintaining normal serum phosphate levels is an important therapeutic goal for dialysis treatment for both acute and chronic renal failure patients[3, 4]. Elevated serum phosphate levels have been associated with congestive heart failure in dialysis patients[5]. In addition, elevated serum phosphate levels or Hyperphosphatemia are associated with higher mortality in dialysis patients with strong relationships to parathyroid hormone and cardiac based causes of death[6].

Specific Aims:

Serum urea concentration in continuous ambulatory peritoneal dialysis[1] and chronic intermittent hemodialysis[2] patients range from approximately 7 to 16 mM and 1 to 55 mM (two standard deviations), respectively. Our urea assay will be developed for a dynamic range of 1 to 65 mM. Serum phosphate concentrations in normal and hemodialysis patients range from approximately 0.8 to 1.4 mM[7] and 0.8 to 2.4mM[8], respectively. Thus we will develop a dynamic range of 0.5 to 3 mM.

Methods and Procedures:

There are two possible ATP-based enzymatic pathways for a urea assay based on bioluminescence: urease/ATP deaminase and urea carboxylase.

There are a number of ATP-based enzyme reactions that could be applied in measuring phosphate (see www.expasy.ch click on Boehringer-Mannheim Metabolic Pathways), including glutamine synthetase.

We will then determine the optimum concentrations of the reagents and enzymes with the goal of maximizing light output and resolution of the assay[9]. The optimum pH of the assays will be determined as different enzymes have different optimum pHs for maximum catalytic activity. Several buffers, including glycine-glycine, TRIS, or Tricine, will be studied. The effect of inhibition of reagents and enzymes from one coupled reaction on the other coupled reactions will be measured.

These methods and protocol will be used in the optimization and development of the urea assay and several of the possible phosphate assays. We will then select the optimum aqueous assay for each analyte for development of the final dry reagent assays.

The lyophilization studies[10] will include storage time (weeks to months) and conditions (temperature and environment), additive and stabilizer concentration and type (including sugars such as sucrose and trehalose, polymers such as dextran, and anti-oxidants), and reconstitution time. Experiments will be conducted with a two-stage lyophilizer.

The effects of various lyophilization additives on firefly luciferase and on the other needed enzymes will be studied, including various antioxidants and additives to increase the glass transition temperature of the amorphous phase of the lyophilized products[10].

We expect to demonstrate the feasibility of bioluminescence-based dry reagent (dip-stick) assays for urea and phosphate by the end of the grant.

The enzymatic reactions will be studied by measuring the light produced by the firefly luciferase reaction and confirmed with appropriate UV/Vis absorbance experiments. The assays will be compared with and validated against existing standard methods. The light will be measured using a photon multiplier tube (PMT) luminometer and/or charge coupled device (CCD) camera.

Preliminary Results:

The urea assay based on urea carboxylase was chosen to bestudied, because ATP deaminase is not commercially available. Furthermore, a bioluminescent assay based on urea carboxylase has been previously developed.

We are currently developing a homogeneous (single-step) urea assay by coupling the urea carboxylase reaction with the firefly luciferase reaction (4):

$$\text{Urea} + \text{ATP} \xrightarrow[\text{K}^+,\ \text{HCO}_3^-,\ \text{Mg}^{2+}]{\text{Urea Carboxylase, E.C. 6.3.4.6}} 2\,\text{NH}_3 + \text{CO}_2 + \text{ADP} + \text{P}_i$$

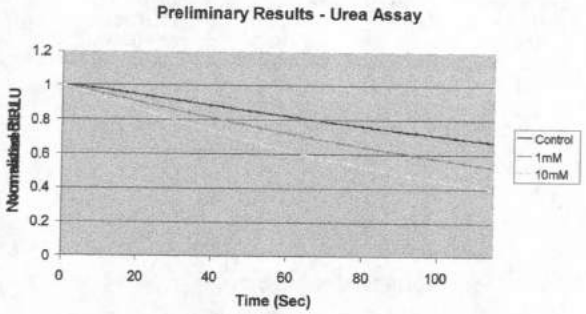
$$\text{Luciferin} + \text{O}_2 + \text{ATP} \xrightarrow[\text{Mg}^{2+}]{\text{Firefly Luciferase, E.C. 1.13.12.7}} \text{Oxyluciferin} + \text{AMP} + \text{Pyrophosphate} + \text{Light} + \text{CO}_2$$

ATP = Adenosine triphosphate
AMP = Adenosine monophosphate

The concentration of urea is determined by the reduction of light output compared with a control containing no urea. The greater the urea concentration, the greater the reduction of light produced by the firefly luciferase reaction, because urea carboxylase competes with firefly luciferase for ATP.

The initial optimization of the assay included reagent concentration determination and buffer selection. The reagents include ATP, luciferin, firefly luciferase, potassium, bicarbonate, and magnesium, and urea carboxylase. The concentration of ATP was set at ~0.1 μM, which is within the approximately steady state light production of the firefly luciferase reaction (well below the Km of ATP [~0.250 mM]). The luciferin concentration was saturated at 0.2 mM (well above the Km of the firefly luciferase for luciferin [2 μM]), so that changes in light production are based solely on the concentration of urea.

The following figure shows some preliminary results for the urea assay.



Budget (First year):

Graduate Research Assistant (R. Davies)	$18,000 + $1620 (benefits)
Supplies	$2,000
Total	$21,620
Indirect Costs (50.5%)	10,918
Total Costs	32,538

Reports: Brief written quarterly reports will be provided, as well as an oral presentation and demonstration. A complete final report and presentation will constitute the fourth quarterly report.

Intellectual Property Issues:

University's Office of Technology Transfer (581-7792). We are working on other industry and University funded biosensors and related technologies. This work has been in development for about 5 years, funded from several sources. We expect that Frezenius will have a right of first refusal for the development of the technologies developed in this program for the dialysis market. We would of course reserve the rights to use the assays developed in this contract for applications outside the negotiated area of application. Prior to beginning the work, the U of Utah and Frezenius will likely into a formal contract clarifying these points.

References:

1. De Deyn, P., *et al.*, *Guanidino compounds in uraemic dialysed patients.* Clin Chim Acta, 1986. **157**(2): p. 143-50.
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5. Stack, A.G. and W.E. Bloembergen, *A cross-sectional study of the prevalence and clinical correlates of congestive heart failure among incident US dialysis patients.* Am J Kidney Dis, 2001. **38**(5): p. 992-1000.
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9. Brolin, S. and G. Wettermark, *Bioluminescence Analysis.* 1992: VCH.
10. Davies, R., R.A. Van Wagenen, and J. Andrade. *Stabilization of firefly luciferase with anti-oxidants: a preliminary study.* in *11th International Symposium on Bioluminescence and Chemiluminescence.* 2000. Monterey, California: John Wiley & Sons.

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A **Preliminary** Proposal to: **Fresenius Medical Care**
Renal Product Technologies
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Date: 5/10/01

From:
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University of Utah
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Phone: 801-581-4379 Fax: 801-585-5361
joe.andrade@m.cc.utah.edu
www.bioen.utah.edu/faculty/jda

Title: **Bioluminescent Assays for Creatinine and Urea: Towards a DialysisChip**

Abstract:

Chronic renal failure patients should be regularly monitored to assure adequacy of dialysis and nutritional status. Bioluminescent assays for creatinine and urea will be developed in a traditional aqueous assay format. These assays will then be modified and developed for dry reagent (dipstick) application using lyophilized reagents for eventual application in a DialysisSensor or RenalSensor. The dry reagents will be reconstituted with the analytical sample (blood, dialysate, urine). Development of the aqueous assays will include optimization of buffer type, pH, reagent concentration, enzyme ratio, enzyme concentration, and other appropriate parameters. The development of the lyophilized or reconstituted assay will consider storage time and conditions, additive/stabilizer type and concentration, and reconstitution time and conditions. The assays will be compared with and validated against existing standard methods.

Background and Significance:

The development of a cost-efficient, home-care, low-sample volume biosensor for creatinine and urea would be useful to peritoneal dialysis and hemodialysis patients as well as to the development of more efficient dialyser cassettes and associated materials. A biosensor for measurement of creatinine and urea in blood would be particularly important for home peritoneal dialysis patients so that the physicians would have serum concentration data without the need for a costly and time-consuming patient visit (1,2). Peritoneal dialysis patients currently compose approximately 15-17% of renal dialysis population. The current push towards more home hemodialysis means a biosensor would offer a needed and convenient service to hemodialysis patients and their physicians. Adding additional chemistry sensing channels to future versions of the biosensor is straightforward.

Specific Aims:

Serum creatinine concentration in continuous ambulatory peritoneal dialysis (1) and chronic intermittent hemodialysis (2) patients range from approximately 500 to 1700 μM and 600 to 1500 μM (two standard deviations), respectively. Our creatinine assay will be developed for a dynamic range of 400 to 1700 μM with at least 5% accuracy. Serum urea concentrations in peritoneal and hemodialysis patients range from approximately 7 to 16 mM and 0 to 55 mM, respectively. Thus we will develop a dynamic range of 0 to 65 mM with at least 5% accuracy.

Methods and Procedures:

There are a number of ATP-based enzyme reactions that could be applied in measuring creatinine (see www.expasy.ch click on Boehringer-Mannheim Metabolic Pathways), including creatinine deaminase/ATP deaminase, creatininase/creatine kinase, and creatinine deaminase/methylhydantoin amidohydrolase.

There are two possible ATP-based enzymatic pathways for a urea assay based on bioluminescence: urease/ATP deaminase and urea carboxylase.

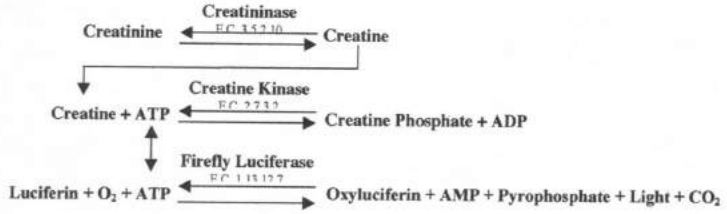
We will model and simulate these pathways using our standard methods for assay development (3,4,6). We will then determine the optimum concentrations of the reagents and enzymes with the goal of maximizing light output and resolution of the assay (5). The optimum pH of the assays will be determined as different enzymes have different optimum pHs for maximum catalytic activity. Several buffers, including glycine-glycine, TRIS, and/or Tricine, will be studied. The effect of inhibition of reagents and enzymes from one coupled reaction on the other coupled reactions will be measured.

The lyophilization studies (6) will include storage time (weeks to months) and conditions (temperature, humidity, and environment), additive and stabilizer concentration and type (including sugars such as sucrose and trehalose, polymers such as dextran, and anti-oxidants), and reconstitution time. Experiments will be conducted with a two-stage lyophilizer.

The enzymatic reactions will be studied by measuring the light produced by the firefly luciferase reaction and confirmed with appropriate UV/Vis absorbance experiments. The assays will be compared with and validated against existing standard methods. The light will be measured using a photon multiplier tube (PMT) luminometer and/or charge coupled device (CCD) camera.

Preliminary Results:

We have preliminarily developed a homogeneous (single-step) creatinine assay by coupling the creatininase and creatine kinase reactions with the firefly luciferase reaction (4):



The concentration of creatinine is determined by the reduction of light output compared with a control containing no creatinine. The greater the creatinine concentration, the greater the reduction of light produced by the firefly luciferase reaction, because creatine kinase competes with firefly luciferase for ATP. A simulation of the homogenous system described above was used to determine starting points for aqueous optimization experiments (3,4).

The optimization of the assay included reagent concentration determination, buffer selection, enzyme ratio, and necessary additives. The reagents include ATP, luciferin, firefly luciferase, creatine kinase, and creatininase. The concentration of ATP was set at ~1 μM, which is within the approximately linear or steady state light production of the firefly luciferase reaction (well below the Km of ATP [~0.250 mM]). The luciferin concentration was saturated at 0.1 mM (well above the Km of the firefly luciferase for luciferin [2 μM]), so that changes in light production are based solely on the concentration of creatinine.

These methods and protocol will be used in the optimization and development of the other two creatinine assays and the two urea assays. We will then select the optimum method for each analyte for development of the final dry reagent assays.

The effects of various lyophilization additives on firefly luciferase and on the other needed enzymes will be studied, including various antioxidants and additives to increase the glass transition temperature of the amorphous phase of the lyophilized products (6).

We expect to demonstrate the feasibility of bioluminescence-based dry reagent (dip-stick) assays for creatinine and urea by the end of the first year of the grant.

Budget (First year):

Graduate Research Assistant (R. Davies)	$18,000 + $1620 (benefits)
Supplies	$2,000
Total	$21,620
Indirect Costs (50.5%)	10,918
Total Costs	32,538

Reports: Brief written quarterly reports will be provided, as well as an oral presentation and demonstration. A complete final report and presentation will constitute the fourth quarterly report.

Intellectual Property Issues:

These will have to be discussed with the University's Office of Technology Transfer (581-7792). We are working on other industry and University funded biosensors and related technologies. This work has been in development for about 5 years, funded from several sources. It would certainly be desirable for Frezenius to have a right of first refusal for their development of these technologies for the dialysis market. This would require negotiating a formal contract for this work (and paying the 50.5% indirect costs). We would of course reserve the rights to use the assays developed in this contract for applications outside the negotiated area of application. These issues can be resolved and included in the formal proposal.

References:

1. De Deyn, P.P. *et al.* Serum guanidino compound levels and clearances in uremic patients treated with continuous ambulatory peritoneal dialysis. *Nephron* **54**, 307-12 (1990).
2. De Deyn, P., Marescau, B., Lornoy, W., Becaus, I. & Lowenthal, A. Guanidino compounds in uraemic dialysed patients. *Clin Chim Acta* **157**, 143-50. (1986).
3. Mendes, P. & Kell, D. Non-linear optimization of biochemical pathways: applications to metabolic engineering and parameter estimation. *Bioinformatics* **14**, 869-83 (1998).
4. J. Smith, R.D., J. Andrade, R. A. Van Wagenen. Optimizing Biosensor Design with Computer Modeling: A Case Study Involving Creatine. in *11th International Symposium on Bioluminescence and Chemiluminescence* Vol. 11 (ed. Kricka, D.L.) (John Wiley & Sons, Monterey, California, 2000).
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6. R. Davies, R.A.V.W., J. Andrade. Stabilization of firefly luciferase with anti-oxidants: a preliminary study. in *11th International Symposium on Bioluminescence and Chemiluminescence* Vol. 11 (ed. Kricka, D.L.) (John Wiley & Sons, Monterey, California, 2000).