ElectroMechanical Membrane-Enabled Transfer (EMMET) System

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Co-inventors

Alberto Giacomello
Keeshan Williams
Carlo Yuvienco

Introduction

With the explosion of proteomics and protein engineering, modern medicine and life science has taken a strong foothold in bench-scale laboratories, both academic and industrial. A vital component of bench-scale development of protein-based technologies is purification. Engineered proteins often undergo a stage of the purification process termed ultrafiltration separation. This involves the separation of the protein of interest from their non-proteinaceous solvent components such as salts (e.g. NaCl) and small molecule denaturants (e.g. DTT, imidazole, urea). This is most commonly accomplished by way of ultrafiltration membrane exchange against a concentration gradient of solutes.

The typical setup consists of protein solutions dialyzed against a bulk buffer solution that is of a lesser concentration of solutes. The volumetric ratios of the bulk to the protein solution are usually prepared between 300:1 and 500:1 (bulk:protein) to ensure timely and complete transfer of solutes from the protein solution to the bulk. The bulk is replenished with fresh solution every 2 hours for up to 8 hours. Often experimenters will allow the exchange of solutes into the final buffer solution to occur for 17 hours to further ensure that the process has gone to completion (Figure 1).

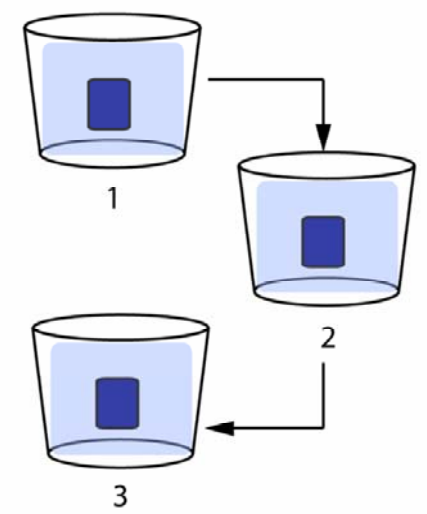


Figure 1: Diagram of typical dialysis procedure

This process, to the best of our knowledge (based on investigations into the academic and industrial community), is not monitored on-line when performed in the average life science laboratory. Attempts have been made to evaluate the structural integrity and activity of enzymes following renaturation by systematic dialysis.[1,2] However, these experimental setups monitored only the structural state of proteins corresponding to various trials of different parameters; no attempts at on-line process monitoring were made.

In addition, the element of control is limited to the discretized changing of the buffer solution and the length of time allowed for exchange across the membrane. Inherent in this process is the question of whether the protein solution is fully dialyzed and thus has come to full equilibrium with the solute concentration of the final buffer solution. Furthermore, the process cannot be optimized for time since there is no time-dependent monitoring of the solute concentrations in the bulk and/or in the protein solution.

Our prototype design aims at addressing this pitfall of contemporary bench-scale protein dialysis by monitoring the dialysis process by measuring changes in solute concentrations and the resultant changes in solution conductivity. In addition, the proposed device will continuously adjust the composition of the bulk buffer solution based on the aforementioned monitoring process.

Theory

Dialysis in Protein Engineering

Ultrafiltration membrane separation is a well-documented chemical engineering phenomenon. In dialysis, "there is little or no pressure difference across the membrane, and the flux of each solute is proportional to the concentration difference. Solutes of high molecular weight are mostly retained in the feed solution, because their diffusivity is low and because diffusion in small pores is greatly hindered when the molecules are almost as large as the pores.[3]" The action of mass transport of small molecules through porous membranes is thus generally governed by Equation 1.[3] Figure 2 illustrates the nature of concentration profiles across a membrane.

Solute flux, $J_A = K_A(c_1 - c_2)$ where K_A = mass transfer coefficient

c_1 = concentration inside membrane

c_2 = concentration of solute in the bulk fluid

Equation 1: General equation for mass transfer across a membrane

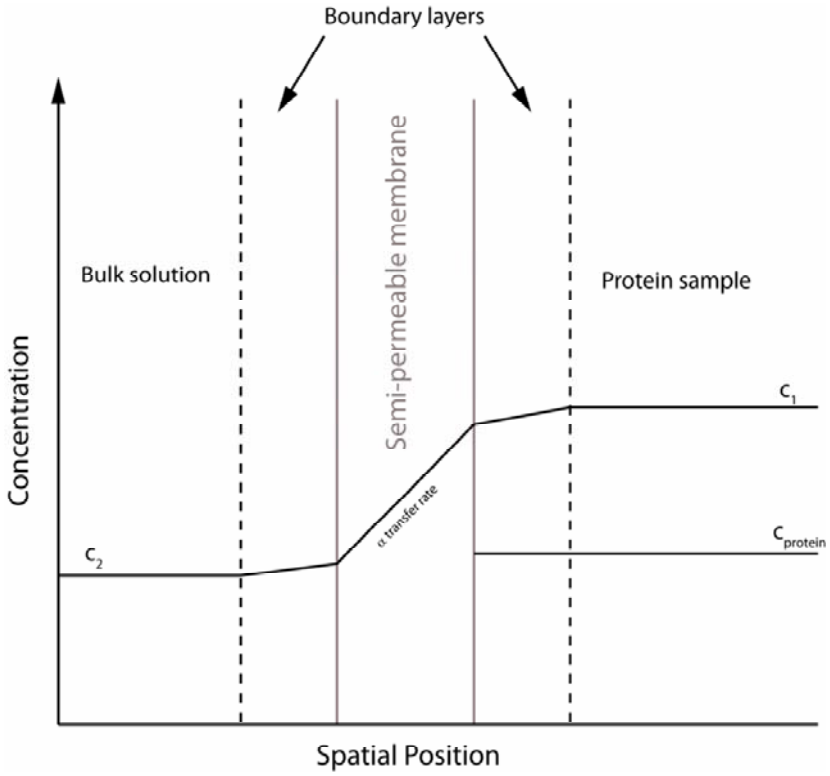


Figure 2: Concentration profile across a semi-permeable membrane

In protein engineering laboratories, dialysis will often be employed to gradually separate denaturants and salts (necessary for prior purification and sample preparation steps) from a protein sample. The gradual control of this process has been documented to be essential for proper folding of some proteins.

In a dynamic system in which the concentrations c_1 and c_2 are allowed to reach equilibrium across the membrane, eventually the flux of solute approaches zero. If the c_2 concentration is dramatically reduced, the gradient may be too steep resulting in an overwhelming outward flux of solutes. The latter case has

been documented to result in the misfolding of proteins retained in the sample solution. This occurs because of the salt-dependent folding kinetics of certain proteins. In the absence of salts, intermediate structures formed during the folding process may undergo intermolecular interactions, leading to protein aggregation and/or precipitation.[4,5] This effect is mitigated by way of step-wise dialysis techniques, in which the bulk solution starts with a known solute (denaturants and salts) concentration relatively close to the solute concentration of the protein sample. Subsequent changes of bulk solution will contain lesser and lesser amounts of starting solutes. Thus this induces a solute flux that is *gradually* discretized across time (Figure 3).

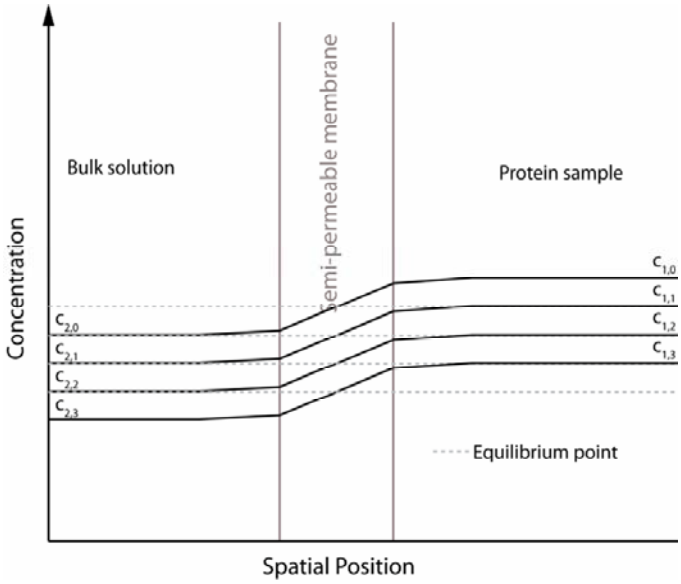


Figure 3: Concentration profile during 4-stage stepwise dialysis

The proposed device allows the continuous variation of the solute bulk concentration with respect to time, thus transforming $c_2(t)$ from a discretized function to a continuous function. This will be accomplished by way of a controlled introduction of fresh diluent into the bulk solution, allowing the bulk solution concentration to be gradually reduced with time (Figure 4a). In addition, the difference in solute concentration difference between the protein sample solution and bulk solution will be monitored with two conductivity sensors – one isolated to the protein solution sample and one isolated to the bulk solution. The sensor information will be used to maintain the solute concentration gradient (and thus its flux) across the membrane.

The EMMET system also aims to save time and material usage. Current laboratory setups typically do not monitor the changing solute concentration of the protein solution and/or bulk solution. These variables thus remain unknown. Current laboratory practice mitigates this condition by allowing the transfer to take place over several hours with the assumption that equilibrium will eventually be reached.

Therefore, it is possible that the transfer of solutes is allowed to occur beyond the necessary time resulting in waste time and productivity (Figure 4b).

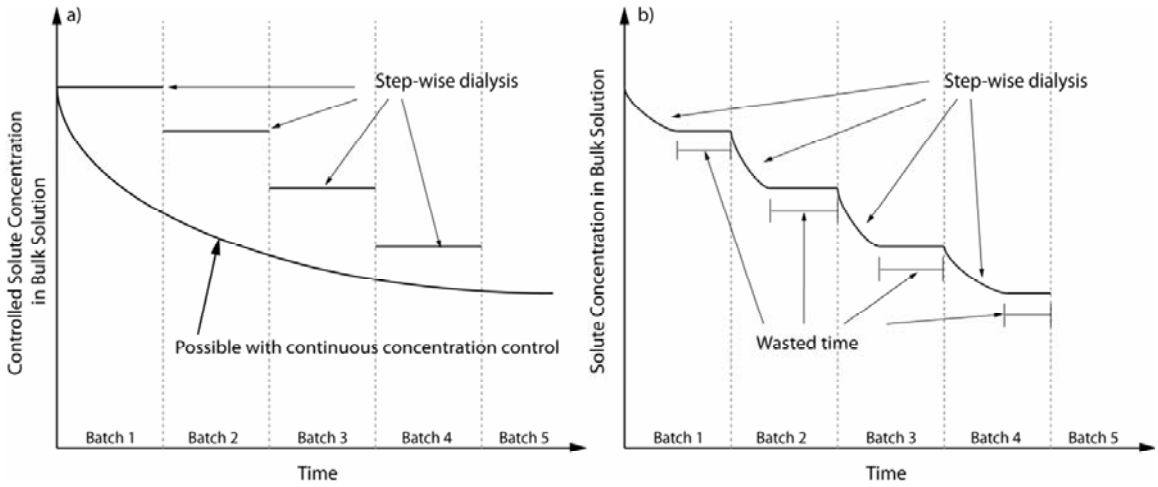


Figure 4: a) Solute concentration control for step-wise dialysis vs. EMMET system b) Potential time savings for an unmonitored step-wise dialysis process

Conductivity and Electrochemistry

In a typical electrical circuit (Figure 5), a flow of electrons that result in a current will occur if there is a potential difference across the terminals of the potential source and the materials of the circuit are sufficiently conductive to allow electrons to pass.

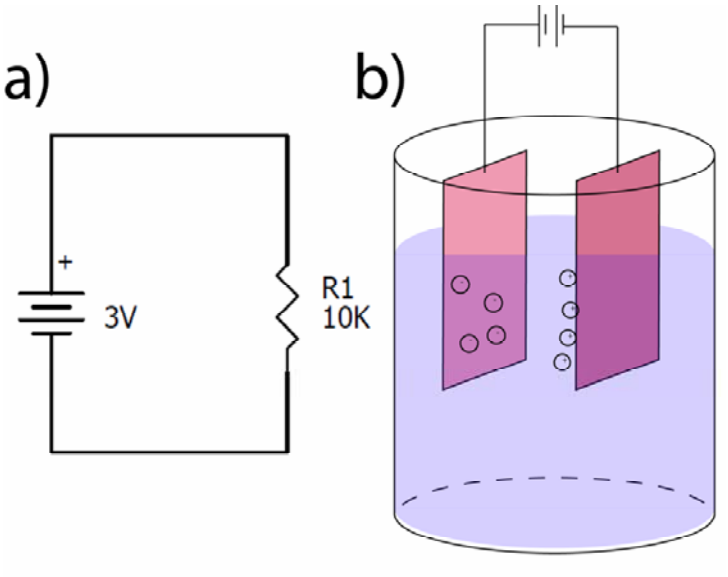


Figure 5: Typical electrochemical circuit

This difference in potential is viewed as the work needed (or that can be done) to move an electric charge form one point in the circuit to another. The work done is given by[6]:

$$\text{Work} = E \cdot q$$

Equation 2: Electric expression for work

where E is the potential difference, given in volts (V) and q is the amount of charge given by the total number of electrons that flow through the circuit, given in coulombs (C). The total amount of charge can be computed by:

$$q = n \cdot F$$

Equation 3: Expression for total charge

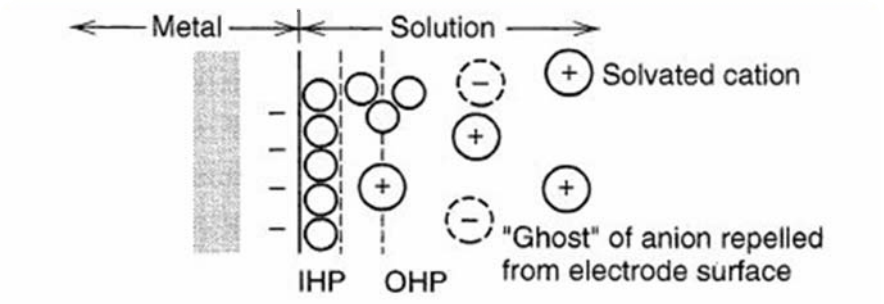
where F is the amount of charge per mole of electrons (Faraday's constant = 9.649×10^4 C/mol of electrons) and n is the amount of moles of electrons. From equation 2, the potential can be viewed as the energy required to "push" electrons through the various elements of the circuit. However, the atomic makeup of the elements of the circuit plays a critical role in how many electrons are allowed to pass (resistance), and is indicated by the measuring the current through the element. The relationship between potential, resistance and current is illustrated according to Ohm's Law[6]:

$$I = \frac{E}{R}$$

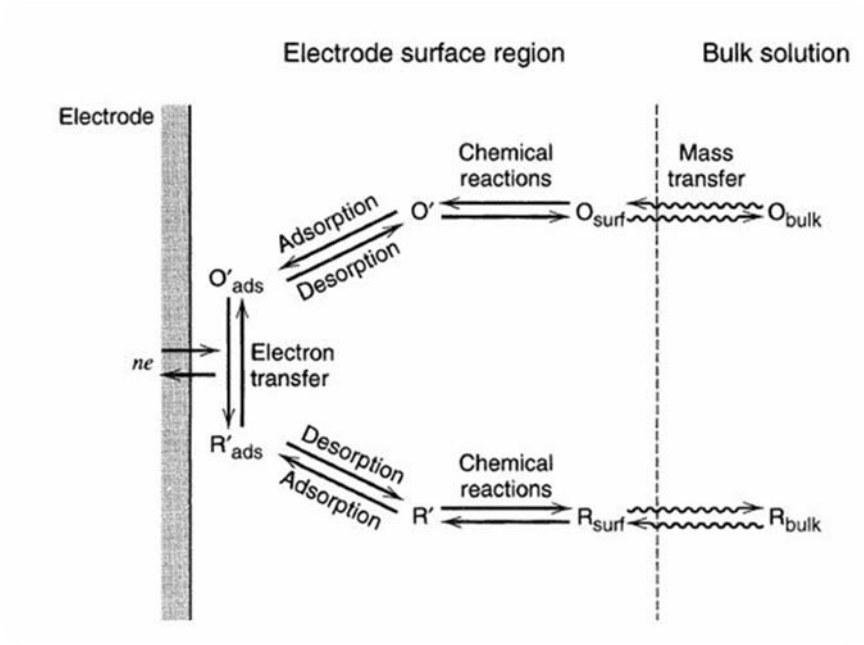
Equation 4: Ohm's Law

where I is current, given in amperes, E is potential (volts) and R is residence, given in ohms. I an electrochemical cell, electrons must pass through a liquid medium, where the species if the medium dictate the respective current.

For the electrochemical circuit to be complete, electrons must cross the interface of the electrical conductor (electrode) and the nonmetallic solution. As such, there must exist sufficient electron carriers (ions) within the solution that allow electrons to be transferred to from one electrode to ion (reduction) and then again from the ions in solution to the other electrode (oxidation) thus completing the circuit (Figure 6a). Therefore the presence of ions in solutions (electrolytes) has a direct effect on the ability of the solution to sustain and electrical current. Ideally, the electrolytic solution can be viewed rudimentarily as any other circuit element, where a potential drop occurs due to its resistance and subsequent current, thus obtaining a measure for the conductivity of the solution. However, due to electrode-solution interactions, such as coupled reduction and oxidation reactions (redox) that occur at the respective electrode interface, obtaining accurate conductivity measurements become somewhat more complicated.



a)



b)

Figure 6: a) Adapted representation of the double layer capacitance that occurs at an electrode interface b) Adapted representation of the general electrode reaction

At the electrode interface, the organization of charged species in solution changes as a function of distance from the surface of the electrode. As a potential is applied to the electrode, a layer of oppositely charged ions begin to aggregate in the immediate vicinity of the electrode surface, in a process that is analogous to the charging of the plates in a capacitor. This initial charged layer attracts a second layer of oppositely charged species that acts like a second capacitor in series. Electrochemically this is referred to as the double-layer capacitance, and is all electrochemical cells. Further away from the electrode surface, the ions can be thought to exist as a homogeneous distribution of positively and negatively charged species, which behaves like a standard resistor. Moreover, redox reaction that may occur can behave as any combination of resistors and capacitors with transient and spatial dependency due to concentration

gradients (Figure 6b). Therefore, to accurate measure the concentration of a particular electro-active species in solution, it is critical that these factors are addressed.

Instrumentation

Experimental Setup

The EMMET system (Figures 7a and 7b) consists of a reaction vessel in which bulk solution surrounds a standard dialysis membrane, typically consisting of regenerated cellulose. The membrane's pores are designed such that only molecules below a certain molecular weight are allowed to freely diffuse across. The protein sample is inserted into the membrane cassette closed to the bulk solution. The bulk solution will initially contain a solute concentration matching that of the protein solution. The bulk solution will be gradually diluted with fresh solvent (free of solutes) that originates from a valve-controlled reservoir located above the reaction vessel. The system currently uses gravity as the driving force for fluid flow, but may be substituted with a standard chemical pump.

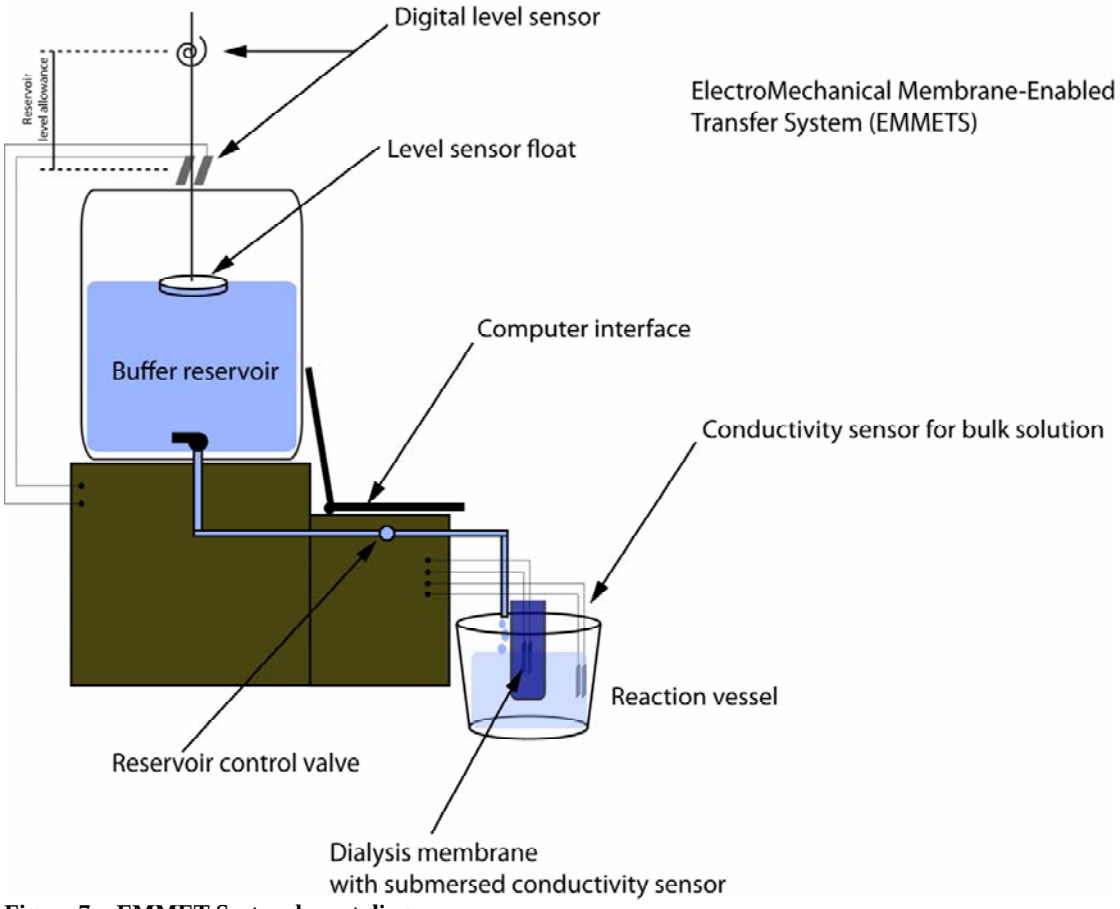


Figure 7a: EMMET System layout diagram

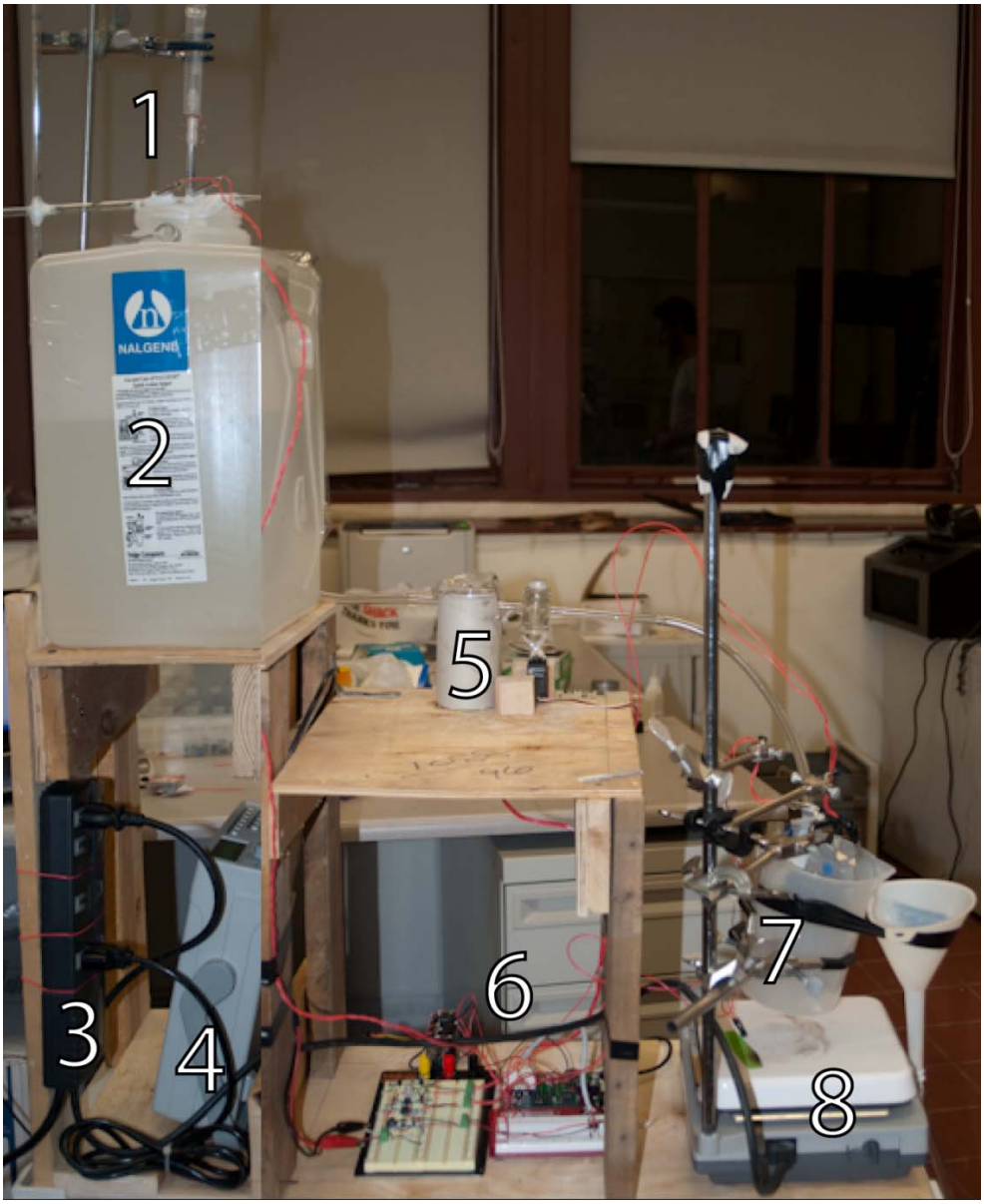


Figure 7b: Actual EMMET prototype setup. 1) Level sensor 2) Bulk reservoir 3) Power strip 4) Signal generator 5) Valve actuator 6) μC and input processing circuitry 7) Reaction vessel 8) Stir plate

Measurement and Control

Two parallel-plate electrodes constructed from aluminum plates (Figure 8a and 8b) will be used to monitor the conductivity of the protein solution and the conductivity of the bulk solution. AC current will be passed across the pair of plates of each electrode, across which the resultant voltage will be measured. The difference in the resultant voltages will be representative of the conductivity gradient, and correspondingly the concentration gradient.

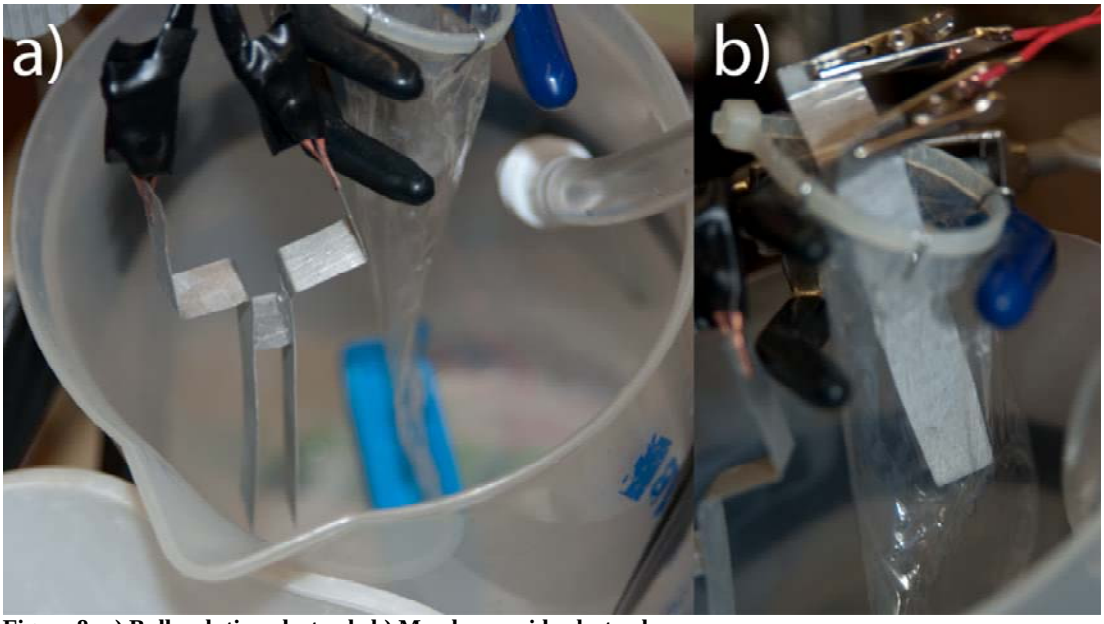


Figure 8: a) Bulk solution electrode b) Membrane-side electrode

This difference will be maintained above an empirically deigned value, such that a concentration gradient persists. Below a *threshold voltage* v_{low}, indicating a (relative) equilibrium state, the valve to the reservoir will be actuated to the open state. Diluent will then spill into the reaction vessel, driving the solute concentration in the bulk down. After some time t_{open}, the reaction vessel will be allowed to mix thoroughly with the fresh diluent for some time t_{mix}. After t_{mix}, if the gradient is still not steep enough to surpass the threshold voltage, the valve will be opened again for another cycle (Figure 9). The end-user will have control of the v_{low}, t_{mix}, and t_{open}, which may vary depending on the reaction vessel size and electrode sensitivity.

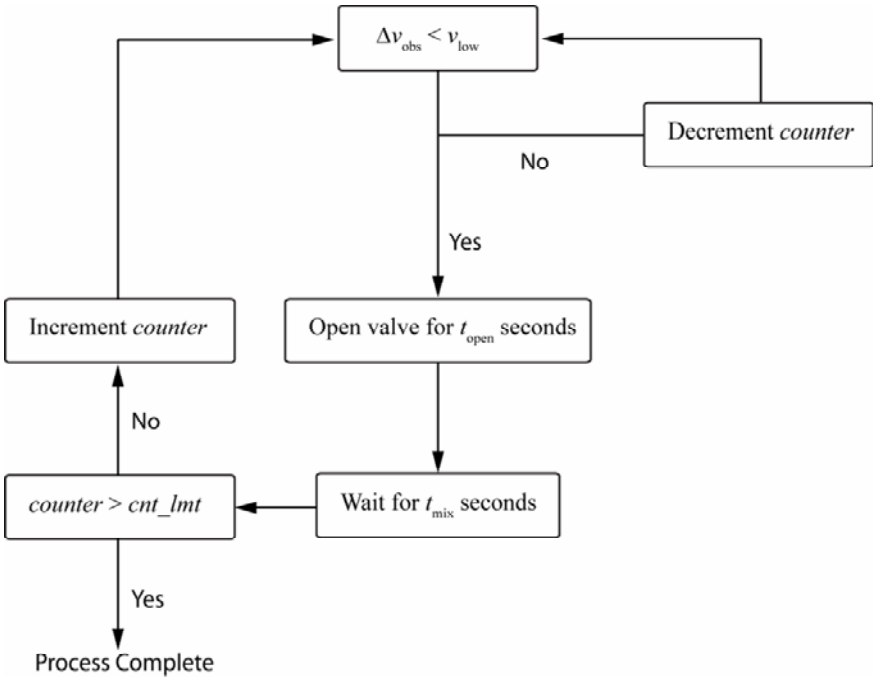


Figure 9: Process control flowchart

The choice of appropriate v_{low}, t_{mix}, and t_{open} is left to empirical determination and the discretion of the end-user. v_{low} should be determined based on the minimum desired concentration gradient to be maintained. A t_{open} value should be chosen such that the reaction vessel bulk solution is not over-diluted, resulting in an excessively sharp concentration gradient. In general, conservative t_{mix} and t_{open} values should be chosen in order to avoid the possibility of gradient overshoot, which would be readily detected by the differential electrode measurements. Overshooting the desired gradient risks applying too sharp of a gradient to the system that may result in protein misfolding.

The variable *counter* will be incremented/decremented to keep track of whether the process has reached completion. The *cnt_lmt* parameter, specified by the end-user, indicates the maximum number of times the system will pulse the reaction vessel with fresh buffer, after which it will be assumed that the protein has reached its final equilibrium concentration – the reservoir's solute concentration.

The glass valve controlling flow from the reservoir to the reaction vessel is actuated via a standard servo (Parallax Inc.). Their mechanical action is coupled in the present prototype by plastic tie straps (Figure 10).

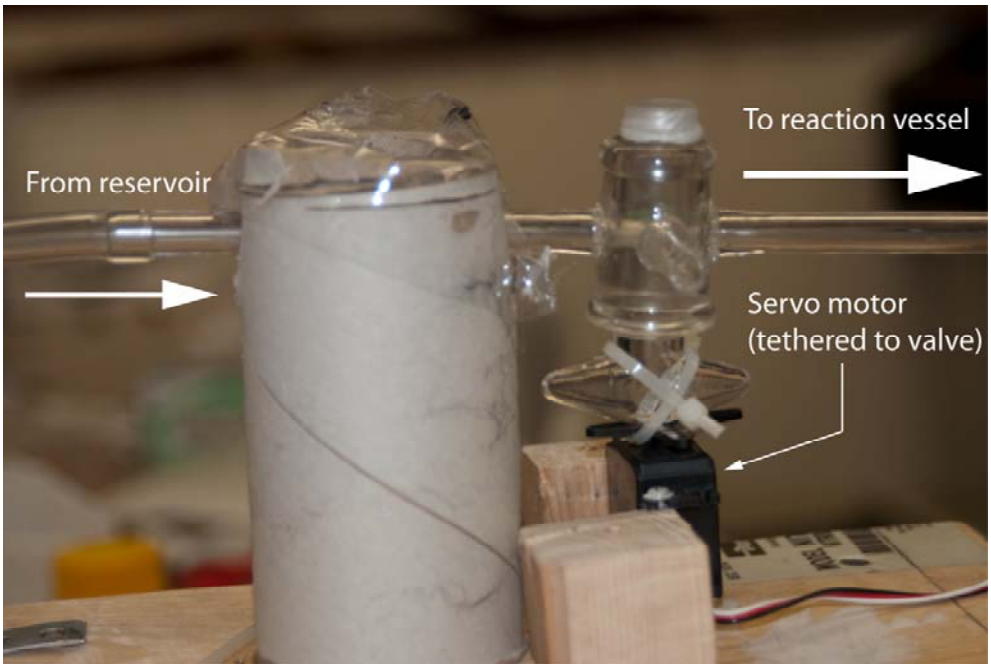


Figure 10: Valve actuator controlling flow from reservoir to reaction vessel

In addition, a digital level sensor was implemented to indicate the depletion of solution from the reservoir, beyond which the system stops actuating the valve and continues to warn the end-user. The digital level sensor consists of a plastic rod, tethered at its based to a float inside the reservoir that descends into the tank with the fluid level. A coil of bare copper wire is tethered to the rod at its opposite end. As the rod descends into the tank, the copper coil also descends and approaches two aluminum contacts (Figure 11), which mimic a normally open button. When the fluid reaches a low enough level, the coil touches both aluminum contacts and thereby closes an active-low switch circuit, which is monitored by the microcontroller.

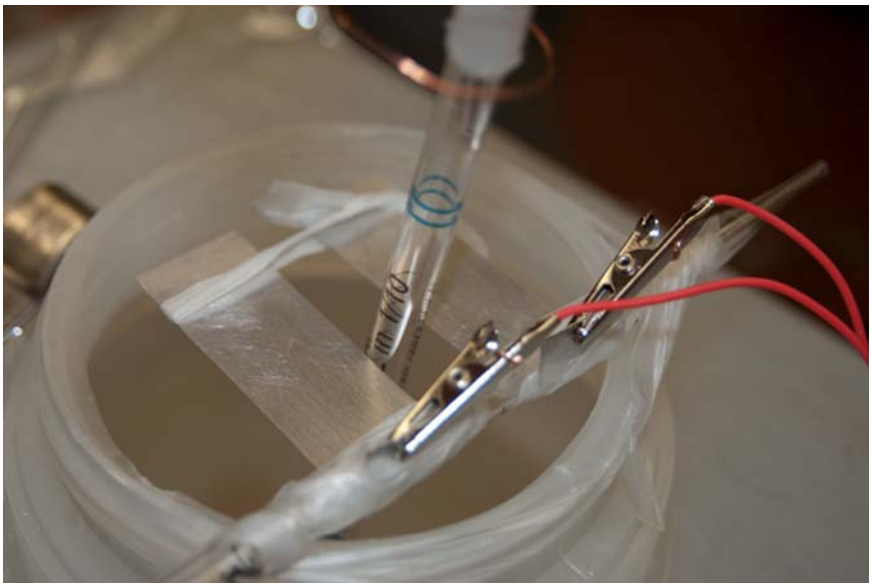


Figure 11: Aluminum contacts of the level sensor that mimic a normally open active-low digital switch

Sensor Design

Water is an electrically insulating media; presence of charge-carrying particles, however, allows current passage through solutions. Depending on the concentration, the type of ions, and reactions taking place, the electric properties of a solution vary, furnishing researchers with a whole range of experimental techniques.

In the simple case, there are no reactions occurring and the electrical behavior of a solution exposed to a voltage difference may be modeled as a resistor and capacitor in series. The dependence of those parameters from concentration is not trivial, and we refer for further details to Bard et al. [6]

The capacitive component is due to the layering of ions on the plates of the electrode, phenomenon that can be avoided in measurements by use of an AC signal with sufficiently high frequency. In this way a direct correlation between concentration and resistance can be obtained, allowing for an effective measurement technique.

However, there are many influential factors confounding the exact determination of concentration from resistance (e.g. temperature, capacitive coupling with walls, depth etc). In our case we found out that bare aluminum electrodes give readable results, while other designs were discarded because of insufficient sensitivity, due most probably to bonding with other metals. We avoided soldering by use of alligator clips, which must be kept dry to maintain correct operation. The final design may be visualized in Figure

8, showing the two electrodes, measuring concentration of the bulk (Figure 8a) and of the sample solution (Figure 8b).

This design is aimed to diminish interaction between solution and the sensors. The presence of current passing in the sample may, in fact, affect the protein function. Furthermore, if the voltage across the electrodes exceeds 0.5V, electrolysis is likely to occur. A possible alternative is to make use of ion selective electrodes (ISE), which do not affect the sample solution. Moreover, ISEs are found on the market, allowing isolated measurement of particular species. In this way protein purification can be performed with the previously described setup for cases different from NaCl. For a review of the possibilities that ISEs offer, in general for analytical chemistry and specifically for this application, one may refer to Koryta.[7] It must be noted that costs would have considerably risen with the adoption of this second method.

Circuit Design

The concentration of ions in solution, as previously more extensively explained, determines the electrical resistance between two electrodes. In particular, to avoid polarization of the solution AC measurements must be made, at conveniently high frequencies. In our case, a campaign of measuring NaCl solution conductivities suggested the frequency of 1kHz and higher.

An ideal way to measure resistance is by mean of Ohm's law, featuring a linear relationship: for this reason a current source was constructed and the information about concentration derived from the voltage drop across each electrode. A function generator drove the current source, whose schematics may be seen in Figure. In a future implementation of the system the design of a cheaper oscillator may be considered. In this prototype of the EMMET system, however, such a generator was more useful due to its flexibility, wide range of operation, and steadiness of output voltage.

After the source-block, the schematics (Figure 12) show an instrumentation amplifier that compares the voltage drops of the two solutions and thus yielding a signal proportional to the concentration gradient across the membrane. This configuration was used for the high input resistance, as well as for the good common mode rejection rate allowed.

The final stage consists in rectifying the signal, in order to interface the microcontroller with the measurement system. A full wave rectifier, featuring four diodes, was used. In order to smooth the

"ripple" in the rectified signal an appropriate capacitor was used. The rectified signal was passed to an 8-bit analog-to-digital converter – ADC0831 – configured with a span of 5V and a V_{ref} of 0V.

A Basic Stamp 2 (BS2) microcontroller (Parallax) was used to receive the input from the ADC0831. In addition, the BS2 was programmed to monitor input from the digital level sensor as well as output control signals to the standard servo actuating the valve.

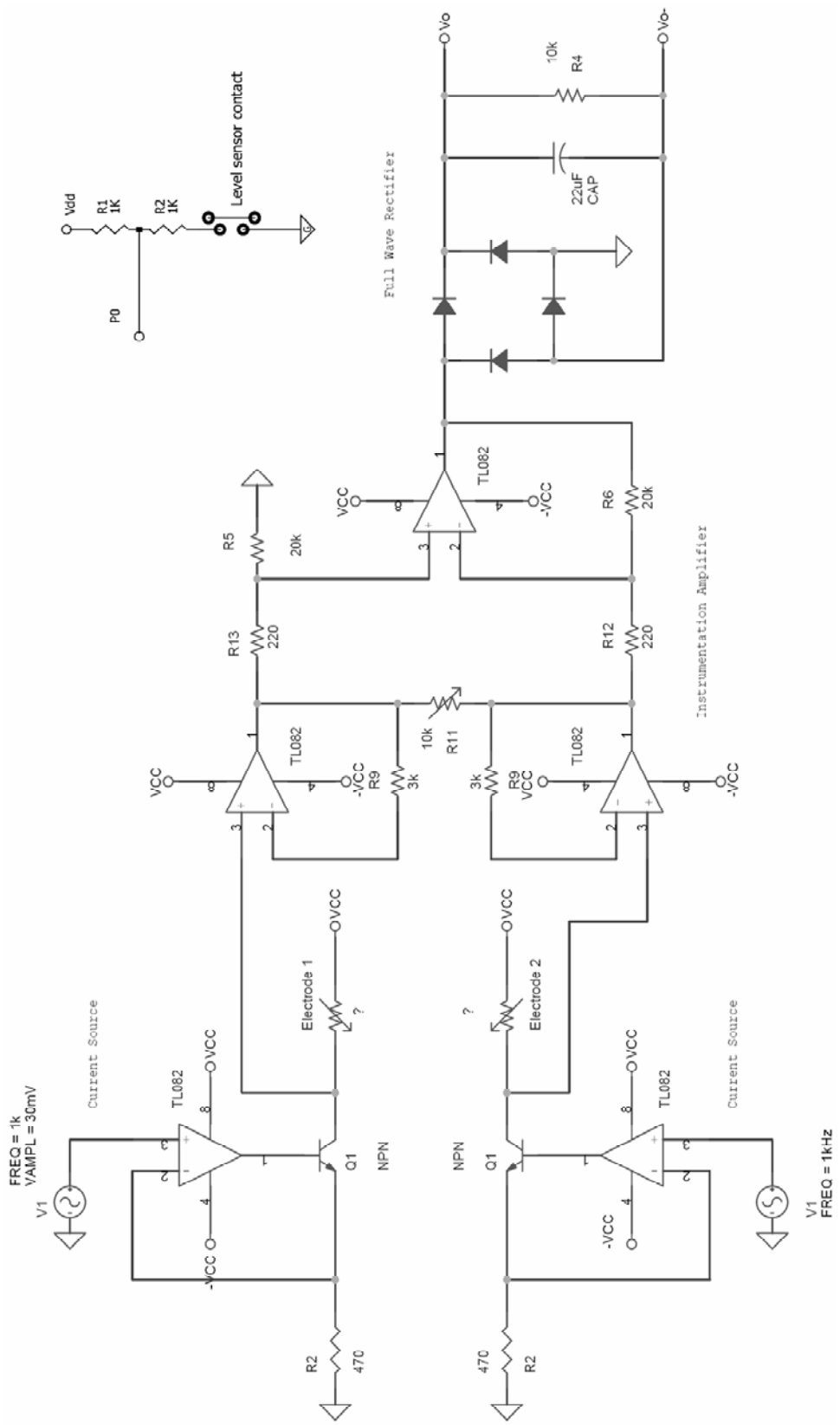


Figure 12: Circuit diagram of amplifier stage, rectification stage, and level sensor

Summary

References

[1] Maeda, Y., Koga, H., Yamada, H., Ueda, T. & Imoto, T. Effective renaturation of reduced lysozyme by gentle removal of urea. *Protein Eng* **8**, 201-205 (1995).

[2] Sorensen, H. P., Sperling-Petersen, H. U. & Mortensen, K. K. Dialysis strategies for protein refolding: preparative streptavidin production. *Protein Expr Purif* **31**, 149-154, doi:S1046592803001335 [pii] (2003).

[3] McCabe, W., Smith, J. & Harriott, P. *Unit operations of chemical engineering*. (McGraw-Hill Science/Engineering/Math, 2004).

[4] Vallejo, L. F. & Rinas, U. Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. *Microb Cell Fact* **3**, 11, doi:10.1186/1475-2859-3-11 1475-2859-3-11 [pii] (2004).

[5] Tsumoto, K., Ejima, D., Kumagai, I. & Arakawa, T. Practical considerations in refolding proteins from inclusion bodies. *Protein Expr Purif* **28**, 1-8, doi:S1046592802006411 [pii] (2003).

[6] Bard, A. & Faulkner, L. *Electrochemical methods: fundamentals and applications.* (Wiley New York, 1980).

[7] Koryta, J. Ion-selective electrodes. *Annual Review of Materials Science* **16**, 13-27 (1986).

PBASIC Code

```
' {$STAMP BS2}
' {$PBASIC 2.5}

'---- Declaration of variables
adcBits VAR Byte
voltin VAR Byte
voltinr VAR Byte
counter VAR Byte

'---- Declaration of constant values for
'     valve/servo positions
valve_open CON 500
valve_close CON 230

'---- Declaration of user input parameters
open_time VAR Byte
pause_time VAR Word
threshold VAR Word
ctr_lmt VAR Byte

'---- Microcontroller pin designations for level
'     sensor, ADC, ANd servo control
LVL PIN 0
CS PIN 1
CLK PIN 2
Datain PIN 3
MTR PIN 14

'---- Subroutine to request and store valve
'     open_time parameter
setup_valve:
  DEBUG "Select valve opening time in s (max 5s)",CR
  DEBUGIN DEC open_time
  IF  open_time < 11  THEN
    open_time= open_time*50
  ELSE
    GOTO setup_valve
  ENDIF

'---- Subroutine to request and store measurement
'     delay parameter - pause_time
setup_pause:
  DEBUG "Select interval between measurements (max 130 s)",CR
  DEBUGIN DEC pause_time
  IF  pause_time < 130 THEN
    pause_time= pause_time*500
  ELSE
```

```
    GOTO setup_pause
  ENDIF

'---- Subroutine to request and store voltage
'     threshold - threshold - which controls the valve actuator
setup_volt:
  DEBUG "Select volt threshold (suggested 2 V)",CR
  DEBUGIN DEC threshold
  IF  threshold  < 5   THEN
    threshold=51*threshold
  ELSE
    GOTO setup_volt
  ENDIF

'---- Subroutine to request and store the limit for the
'     number of times for the system to attempt to establish
'     a solute gradient against an equilibrium
setup_ctr_lmt:
  DEBUG "Select counter limit (suggested > 10)",CR
  DEBUGIN DEC2 ctr_lmt

'---- Main program loop
DO
  PAUSE pause_time
  DEBUG CLS

  '---- Section of main loop that checks the input from
  '     the level sensor and halts program flow if sensor
  '     is activated
  level_check:
  IF LVL = 0 THEN
    DEBUG "Level of diluent low, refill the reservoir", CR, BELL
  GOTO level_check
  ELSE
    DEBUG "Level: OK ", CR
  ENDIF

  '---- Formatted display of ADC output
  GOSUB ADC
  DEBUG "Electrode Potential Difference: ", DEC voltin, ".", DEC2
voltinr, " V", CR

  '---- Conditional statement based on ADC output and
  '     threshold parameter that either results in valve
  '     actuation OR no valve actuation.
  IF adcBits < threshold THEN
    DEBUG "Diluting", CR
    counter = counter + 1
    GOSUB open
  ELSE
    DEBUG "No dilution needed", CR
    counter = 0
```

```
  ENDIF

  '---- Conditional statement to check if counter limit
  '     is reached, indicating completion of dialysis process.
  IF counter > ctr_lmt THEN
    DEBUG CR, CR, "DIALYSIS COMPLETE"
  ENDIF

  DEBUG HOME
LOOP

'---- Subroutine to enable the ADC, collect, and store data
ADC:
  HIGH CS
  LOW CS
  LOW CLK
  PULSOUT CLK, 210
  SHIFTIN Datain, CLK, MSBPOST, [adcBits\8]
  voltin=5*adcBits/255
  voltinr=5*adcBits//255
  voltinr=voltinr*100/255
RETURN

'---- Subroutine to control the position of the servo and
'     thereby actuate the reservoir valve
open:
    DEBUG CR, "Opening valve"
    FOR counter = 0 TO open_time
      PULSOUT MTR, valve_open
      PAUSE 20
    NEXT

    DEBUG CR, "Closing valve"
      FOR counter = 0 TO 150
        PULSOUT MTR, valve_close
        PAUSE 20
      NEXT
RETURN
```