INTRODUCTION TO BIOSEPARATIONS

Affinity Adsorption

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Bioseparation processes have long been regarded as the critical factor in the commercial development of biotechnology. Chemical engineering operations have been widely used to obtain bioproducts, but there are some new separation techniques that enhance these operations that need to be included in chemical engineering courses. Among these techniques, affinity adsorption is of particular interest. Highly valuable proteins such as enzymes, hormones, antibodies, and interferons are obtained through the use of this technique.

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We have developed some basic experiments that permit the demonstration of affinity adsorption techniques to chemical engineering undergraduate students. In our experience, this material is suitable for inclusion in a required senior-level unit operations course since most undergraduates have little exposure to adsorption in general and probably no exposure to protein separations methods in particular.

In the experiments, a dye-ligand affinity adsorbent is used because it is didactic and has management advantages. Dye affinity adsorption is widely recommended and used for large-scale protein purification because dye-ligand adsorbents are cheap, stable, and versatile.[1,2]

Affinity adsorption processes can be used in the concentration of a desired product from a diluted solution or to separate a mixture of solutes using column chromatographic techniques.[3] In some cases, it may be suitable to perform affinity adsorption in a batch-stirred tank.

Much of the information needed to evaluate column performance is contained in typical plots of effluent concentration versus time or breakthrough curve. This curve can be used to determine 1) how much of the column capacity has been used, 2) how much solute is lost in the effluent, and 3) the processing time. This is precisely the performance information needed to optimize processing.[6] Concentration profiles for batch systems can be used in an analogous way.

In affinity adsorption systems after the adsorption step, non-adsorbed material is washed off with equilibrium buffer, and adsorbed compounds are then eluted.[4] Recovery is usually effected by changing the pH, ion strength, or chemical composition of the buffer.[5]

To predict the performance of affinity adsorption systems, theory and experiments must be combined. This article de-

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scribes batch and fixed-bed experiments to demonstrate this predictive approach to undergraduate students.

THEORY

The interaction of proteins with immobilized dyes varies and, for the most part, is not well understood. To describe these complex interactions, a simplified model is often used.[7] The model is the second-order reversible interaction where the protein (P) is assumed to interact with the ligand (D) by a monovalent interaction that has a characteristic binding energy, forming the dye-protein complex (PD) as

$$P + D \Leftrightarrow P \cdot D \tag{1}$$

The adsorption rate for this type of interaction is given by

$$\frac{dq}{dt} = k_1 c(q_m - q) - k_{-1} q \tag{2}$$

where

c = protein concentration in the liquid, ML^{-3}
q = protein concentration in the adsorbent, ML^{-3}
q_m = maximum adsorption capacity of the adsorbent, ML^{-3}
k_1 = forward kinetic constant, $M^{-1}L^3t^{-1}$
k_{-1} = reverse kinetic constant, t^{-1}
t = time, t

The kinetics constants k_1 and k_{-1} are lumped parameters that reflect the contributions of mass transport and binding kinetics as well. At equilibrium, Eq. (2) reduces to the familiar Langmuir isotherm model

$$q^* = \frac{q_m c^*}{K_d + c^*} \tag{3}$$

where * denotes equilibrium concentrations and $K_d = k_{-1}/k_1$ is the dissociation constant.

BATCH MODEL

We will use an expression for the protein uptake by affinity adsorbent in a well-stirred tank initially filled with a protein solution at an initial concentration c_o. The total volume of the system is V and the liquid volume is εV, where ε is the batch void fraction. Then the adsorbent volume is $(1-\varepsilon)V$. Protein concentration in solution at time t is given by[8]

$$c = c_o - \frac{(1-\varepsilon)}{\varepsilon}\left[\frac{(b+a)\left(1-\exp\left\{-\frac{2a(1-\varepsilon)}{\varepsilon}k_1 t\right\}\right)}{\left(\frac{b+a}{b-a}\right)-\exp\left\{-\frac{2a(1-\varepsilon)}{\varepsilon}k_1 t\right\}}\right] \tag{4}$$

where

$$a^2 = b^2 - \left[\frac{C_o \varepsilon}{(1-\varepsilon)}\right]q_m^s \quad \text{and} \quad b = \frac{1}{2}\left[\frac{C_o \varepsilon}{(1-\varepsilon)} + q_m^s + \frac{K_d \varepsilon}{(1-\varepsilon)}\right]$$

and q_m^s is the maximum adsorbent capacity referred to the settled volume of adsorbent.

FIXED-BED MODEL

The model used to describe fixed-bed affinity adsorption is based on isothermal sorption of a single solute in plug flow through a packed bed of uniform spherical particles having a radius r_a, which has a uniform cross-sectional area A of length L and a void fraction ε. Liquid flows through the bed at a superficial velocity of υ, and the column is initially devoid of solute. At time zero the inlet concentration of solute in the mobile phase to the column is changed to c_o.

It has been shown[6] that when axial dispersion is negligible, column mass balance can be expressed as

$$\varepsilon\frac{\partial c}{\partial t} = -\upsilon\frac{\partial c}{\partial z} - (1-\varepsilon)\frac{\partial q}{\partial t} \tag{5}$$

The analytic solution to Eqs. (2) and (5) was first obtained by Thomas[9] and can be expressed as[10]

$$\chi(t,L) = \frac{J\left(\frac{n}{r}, n\Gamma\right)}{J\left(\frac{n}{r}, n\Gamma\right) + \left[1 - J\left(n, \frac{n\Gamma}{r}\right)\right]\exp\left[\left(1-\frac{1}{r}\right)(n-n\Gamma)\right]} \tag{6}$$

where

$$\chi(t,L) = \frac{c}{c_o} \tag{7}$$

$$r = 1 + \frac{c_o}{K_d} \tag{8}$$

$$n = q_m^s k_1 L \frac{A}{F} \tag{9}$$

$$\Gamma = \left(\frac{K_d + c_o}{q_m^s}\right)\left(\frac{Ft}{AL} - \varepsilon\right) \tag{10}$$

and J is a two-parameter function of α and β given by[11]

$$J(\alpha,\beta) = 1 - e^{-\beta}\int_0^{\alpha} e^{-\xi} I_0\left(2\sqrt{\beta\xi}\right)d\xi \qquad (11)$$

where I_0 refers to a zero-order modified Bessel function of the first kind. According to Thomas, when the product of α and β is greater than 36, $J(\alpha,\beta)$ can be calculated within 1% accuracy by

$$J(\alpha,\beta) = \frac{1}{2}\left\{1 - \text{erf}\left(\sqrt{\alpha} - \sqrt{\beta}\right) + \frac{\exp\left[-\left(\sqrt{\alpha} - \sqrt{\beta}\right)^2\right]}{\sqrt{\pi}\left[\sqrt{\beta} + (\alpha\beta)^{\frac{1}{4}}\right]}\right\} \qquad (12)$$

RESOLUTION

Resolution is a measure of the attained separation of two solutes. The resolution of two solute peaks R_s is defined as the distance between the peaks divided by the sum of their average width.

$$R_s = \frac{2(t_B - t_A)}{(W_A + W_B)} \qquad (13)$$

where

t_A, t_B = retention times of components A and B (min)
W_A, W_B = widths of peaks A and B (min)

EXPERIMENTAL PROCEDURE

MATERIALS AND GENERAL PROCEDURES

To conduct the experimental procedures, the adsorbent used was DyeMatrex™ Blue-A (Cibacron Blue F3GA covalently attached to cross-linked 6% agarose) with a particle diameter between 50 to 150 μm purchased from Amicon. Bovine serum albumin (BSA) and lysozyme were the proteins used for adsorption and selectivity experiments, and for void volume determinations, Blue Dextran was employed. These chemicals are available from Sigma and Pharmacia.

All solutions were buffered with 20 mM of sodium phosphate, pH 7. Known volumes of adsorbent were previously prepared by a laboratory assistant by allowing a suspension of adsorbent in 0.5 M of sodium chloride in 20 mM sodium phosphate buffer to settle in a measuring cylinder overnight. A 50:50 (v/v) suspension was then prepared by adjusting the liquid volume to equal that of the settled adsorbent. Air bubbles were removed from the slurry by applying vacuum for ten minutes. Before use, the adsorbent was washed with 20 mM sodium phosphate adsorption buffer.

Protein concentration was estimated by absorbance at 280 nm. Extinction coefficient of 0.56 AU-ml/mg for albumin and 2.16 AU-ml/mg for lysozyme, determined previously by a calibration curve, were given to students. All the experiments were run at room temperature (25 °C).

EQUILIBRIUM EXPERIMENT

The students conducted adsorption equilibrium experiments in the stirred-batch system shown schematically in Figure 1. A typical

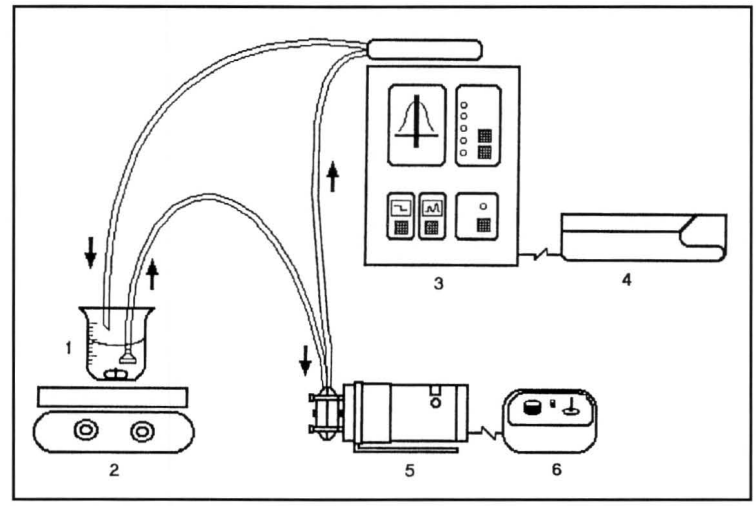

Figure 1. *Apparatus for batch stirred tank experiments: (1) batch adsorption vessel; (2) stirred magnetic plate; (3) UV monitor; (4) chart recorder; (5) peristaltic pump; (6) speed control.*

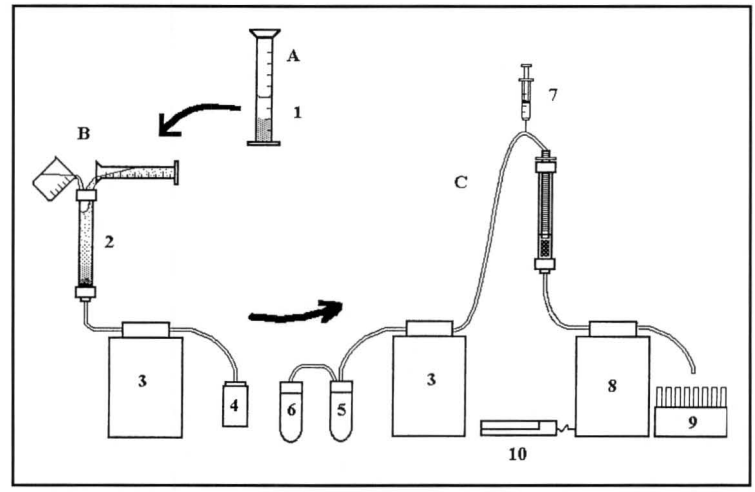

Figure 2. *(A) Adsorbent preparation; (B) Column packing; (C) Experimental arrangement for column experiments. (1) measurement cylinder; (2) column (3) peristaltic pump; (4) vessel; (5) starting buffer or feed vessel; (6) final buffer vessel; (7) sample application; (8) UV monitor; (9) fractions collector; (10) chart recorder.*

experiment consisted of the addition of 2 ml of a 50:50 (v/v) suspension of the adsorbent to 25 ml of buffer in the adsorption vessel. The experiment was conducted by step-wise addition of pulses of a 10 mg/ml BSA solution to the adsorption vessel. At time zero, a 100 µl pulse was added to the batch adsorber. Soluble phase protein concentration was continuously monitored by recycling the liquid phase through a 20 µm porosity net filter adapted to the tubing and through a continuous flow BioRad UV spectrophotometer, using a flow-rate of 1 ml/min. The adsorption was carried out until no appreciable change was observed in the chart recorder connected to the spectrophotometer; this reading was taken as an equilibrium point. Successive pulses were added to the same system to obtain higher equilibrium points until adsorbent saturation was reached. The amount of protein adsorbed at each point was then calculated by mass balances. These values were used to obtain the adsorption isotherm for the system.

KINETICS OF BATCH ADSORPTION EXPERIMENT

The students did batch experiments in the same experimental arrangements (Figure 1). Initially, 25 ml of BSA solution at known concentration (*e.g.,* 0.5 mg/ml) was placed in the well-stirred reactor (50 ml volume). At time zero, 2 ml of a 50:50 (v/v) adsorbent suspension was added to the reactor. Protein concentration was continuously monitored with the spectrophotometer using a recycle flow of 6 ml/min. The adsorption was carried out until no appreciable change was observed.

FIXED-BED EXPERIMENT

Fixed-bed experiments were carried out in a BioRad Chromatographic Econosystem, shown schematically in Figure 2. The adsorbent was measured (Figure 2A) as described above, and then students packed the column (Figure 2B). The breakthrough curve for the system was determined by continuously loading protein to the packed bed until the protein outlet concentration was equal to protein inlet concentration, c_o.

Fixed-bed experiments were performed with 2 ml (settled volume) of adsorbent packed in a chromatographic column having a diameter of 10 mm. Inlet protein solution with a concentration of 1 mg/ml (c_o) was applied using a volumetric flow-rate of 0.3 ml/min. The optical density at 280 nm of the outlet stream was continuously recorded (Figure 2C). Data were plotted in the form of normalized concentration, c/c_o, of the outlet stream against the time of operation. Time zero was taken as the point at which the adsorbate solution first entered the bed. The void volume of the bed was estimated by a pulse of Blue Dextran.

COLUMN SELECTIVITY EXPERIMENT

The experimental arrangement shown in Figure 2 was also used by the students in the selectivity experiment. To measure the column selectivity, a 100 µl pulse of a 4.0 mg/ml albumin and a 1.5 mg/ml of lysozyme solution was applied to the column and then eluted using a linear gradient 0.0-2.0 M NaCl (240 min) in 20mM sodium phosphate buffer. A flow of 0.3 ml/min was maintained throughout. The experiment was performed using an automatic gradient formed program.

SIMULATION STUDIES

Computer programs were developed by students to simulate batch concentration profiles and the breakthrough curve. All computer programs were run using MathCad version 4.0 in a personal computer.

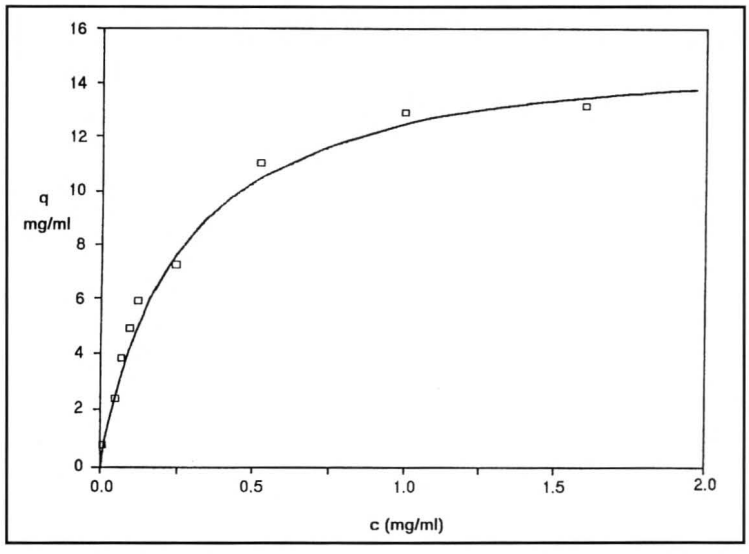

Figure 3. *Adsorption isotherm for bovine serum albumin (BSA) adsorption to Cibacron Blue adsorbent: □ Experimental points; — best-fit to Langmuir model.*

RESULTS

EQUILIBRIUM EXPERIMENT

Equilibrium points were derived from the protein concentration attained at each adsorption step and the corresponding mass balances. With the calculated values, the isotherm for the system shown in Figure 3 is obtained. Equilibrium data were fitted to the Langmuir model (Eq. 3) by nonlinear least-squares regression analysis to obtain the maximum adsorption capacity q_m = 15.6 mg of BSA/ml of adsorbent settled volume and the dissociation constant K_d = 0.25 mg/ml. The solid lines in Figure 3 represent the Langmuir isotherm, which best fits the data. In general, the model fits the data well. These results are well reproduced by students when they measure solution pulses in a proper form.

KINETICS OF BATCH ADSORPTION EXPERIMENT

Batch kinetic experimental results obtained by instructors are shown in Figure 4. The experimental data (small squares) were fitted by the proposed model (Eq. 4) using K_d and q_m values from equilibrium experiments and a k_1 value of 0.0038 ml/mg-s that best fit the kinetic data. The solid line is the best fit to the model. The same approach is used by students for experimental data treatment. The experiment has a high degree of reproducibility.

FIXED-BED EXPERIMENT

A breakthrough curve obtained by instructors for fixed-bed adsorption of BSA is shown in Figure 5. The continuous line in the figure was obtained by fitting the experimental data to the kinetic model in Eq. (6). A value of 0.0009 ml/mg-s for the lumped forward rate constant k_1 was estimated by the best fit to the early part of the curve (that of most interest) using a K_d value of 0.25 mg/ml and the q_{max} of 15.6 mg/ml as determined in batch uptake experiments. A void bed volume of 0.42 as determined with Blue Dextran was used in these simulations. For student's data treatment, the void bed value is provided by instructors. In order to get a good reproducibility of these results by students, special attention must be taken in column packing.

In general, good agreement was observed between theory and experiments, particularly in the early part of the curve. Systematic discrepancies were found in the latter stage of the breakthrough curves, however, where experimental data trail behind the model. These results indicate that the adsorption rate was slower at this latter stage than it was at the beginning of the breakthrough. If a more detailed description of the phenomena is needed, a more sophisticated model should be employed.

It has been pointed out that the kinetic model works better for situations where mass transfer effects are small.[7] This may explain why fit in batch is better than in fixed-bed experiments. For this reason, no attempt was made to predict the kinetic forward rate constant k_1 from the batch experiments.

SELECTIVITY EXPERIMENT

Figure 6 shows selectivity experimental results obtained by instructors. The retention time for BSA is about 70 min and for lysozyme is 180 min. Under the experimental conditions employed, lysozyme binding to the adsorbent is stronger than BSA binding. Both peaks are well developed and about the same size because the difference in the extinction coefficients is well compensated by sample concentration. The experimental resolution obtained in this separation is about 1.87. Correct column packing and sample application allow students to develop these same concentration profiles.

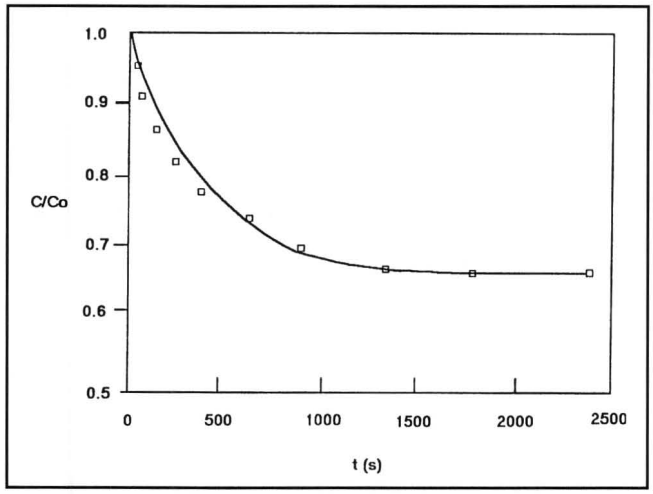

Figure 4. *Bovine serum albumin (BSA) batch adsorption kinetics on Cibacron Blue-ligand adsorbent: □ Experimental points; — Best-fit to batch model.*

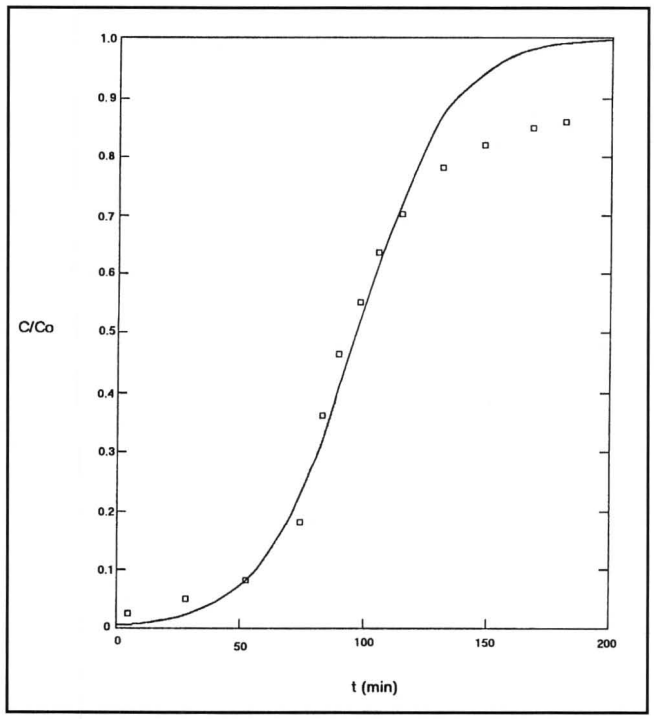

Figure 5. *Fixed-bed breakthrough curve for bovine serum albumin adsorption to Cibacron Blue-ligand adsorbent: □ Experimental points; —Best-fit to kinetic model.*

DISCUSSION

These experiments are of great educational interest because they allow the study of affinity adsorption, a novel concept for chemical engineers. A major advantage of these experiments is the simplicity of the laboratory procedures. The continuous protein concentration monitoring in the experimental systems offers several advantages, permits students to focus on the experiment rather than in taking samples, and requires less supervision.

Small-scale experiments are easier to conduct. Less time and fewer materials are used than in some of the pilot-scale experiments commonly used in traditional chemical engineering laboratories. Small-scale experiments also permit the student to focus on fundamentals rather than on routine procedures.

The experiments can be integrated in three modules: (1) equilibrium and batch experiments; (2) fixed-bed experiments; (3) selectivity experiments. Initial preparation for each module requires less than thirty minutes for the students or laboratory assistant. The experimental measurements for each of the first two modules can be made in approximately three hours; the third module requires about four hours. These experimental modules may be conducted simultaneously if sufficient equipment is available.

The general nature of the experiments should also be emphasized. The procedures here described can be employed with other types of adsorbent-protein systems as well as to work out various situations of interest under different conditions: sample concentration, sample volume, gradient, column length, buffer flow rate, and type of elution buffer.

To evaluate experimental achievements, students are required to submit a report with their experimental results. The report must include a parametric analysis, done with the fixed-bed experimental model, on the influence flow rate and inlet concentration have on column operation capacity. We highly recommend using one of the simulated runs to validate the experimental model.

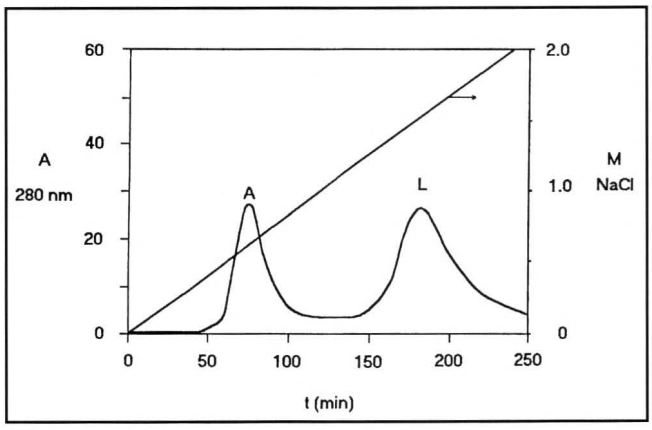

Figure 6. *Elution pattern for lysozyme and albumin mixture on Cibacron-Blue adsorbent column using a linear gradient 0.0 - 2.0 M NaCl (240 min). A flow rate of 0.3 ml/min was maintained throughout. (A) albumin, (L) lysozyme.*

CONCLUSIONS

The BSA-dye adsorbent experimental system allows one to demonstrate affinity adsorption techniques. By combining a simple kinetic model with experimental results, some of the key factors that affect affinity adsorption systems are better grasped by the students. Through this approach, the interrelation between equilibrium and kinetic data—and how to integrate theory and experimental results to predict systems performance—can be emphasized. Very complex phenomena can be demonstrated using a simple and low-cost system that permits the development of textbook-type graphics.

ACKNOWLEDGMENTS

This work has been supported by the Universidad de Sonora and the Consejo Nacional de Ciencia y Technología (CONACyT: 2046-A9302).

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