Alkaline hemolysis fragility is dependent on cell shape: results from a morphology tracker

Cristian Ionescu-Zanetti¹, Lee-Ping Wang¹, Dino Di Carlo¹, Paul Hung¹, Andrea Di Blas², Richard Hughey², and Luke P. Lee¹

(1) Bioengineering Dept., University of California, Berkeley
(2) Computer Engineering Dept., University of California, Santa Cruz

Background: Viral capsids, bacterial toxins, alkaline solutions, and other toxins lyse cells by membrane poration driven colloid-osmotic lysis, which has been studied in detail by averaging over cell populations. However, no quantitative data is available for the changes in the shape and size of individual cells during membrane poration driven colloid-osmotic lysis, or for the relationship between cell shape and fragility.

Methods: Hydroxide, a porating agent, was generated in a microfluidic enclosure containing red blood cells (RBCs) in suspension. Automatic cell recognition, tracking and morphometric measurements were done using a custom image analysis program. Cell area and circular shape factor (CSF) were measured over time for individual cells.

Results: The average CSF goes through a first period of fast increase, corresponding to the conversion of discocytes to spherocytes under internal osmotic pressure, followed by another period of slow increase until the fast lysis event. For individual cells, the minimum CSF is shown to be inversely correlated with cell lifetime (linear regression factor R = 0.44), with discocytes surviving longer than spherocytes. The inflated cell volume to surface area ratio is also positively correlated with lifetime (R = 0.43), while it is uncorrelated with the CSF. Lifetime is best correlated to the ratio of cell inflation volume (V_{final} - V_{initial}) to surface area (R = 0.65).

Conclusions: RBCs are shown to inflate at a rate proportional to their surface area, in agreement with a constant flux model, and lyse after attaining a spherical morphology. Spherical RBCs display increased alkaline hemolysis fragility (shorter lifetimes), providing an explanation for the increased osmotic fragility of RBCs from patients suffering from spherocytosis.

Key Terms: erythrocyte; spherocytosis; alkaline lysis; cell tracking; morphometric analysis, colloid-osmotic lysis, parallel computer acceleration

Hemolysis dynamics in the presence of porating agents is an important area of research, due to the gamut of both natural and synthetic toxins that affect cell death through membrane permeabilization (1). Poration driven hemolysis is intrinsically dependent on the osmotic fragility of the red blood cells (RBCs), since membrane poration results in increased internal osmotic pressure by the colloid-osmotic mechanism, which in turn leads to cell lysis (2). Osmotic fragility itself has been studied extensively and is being used as a diagnostic tool for blood disorders such as spherocytosis and thalassemia. It has been shown that the osmotic fragility of spherocytic RBCs is higher than that of normal shaped RBCs (3). However, it is not clear if RBCs involved in spherocytosis are also different in other ways from normal RBCs, with factors other than cell shape influencing osmotic fragility. As for the relationship between normal cell shape and...
resistance to membrane porating agents (i.e. longer lifetime in presence of the agents), no evidence has been presented to date. Here we address this question by measuring shape changes for individual RBCs before lysis in alkaline media.

It is generally accepted that RBCs swell before bursting after the application of membrane porating agents (2,4). However, observations to date result from either qualitative classification of fixed cells imaged by scanning electron microscopy (5) or population averages obtained from forward angle scattering (6) or dielectric (7) measurements.

Our aim was to directly observe a large number of single RBCs in suspension and measure morphological change leading to lysis. Hydroxide was used as the membrane porating agent and applied to the cells inside a microfluidic device. Recently, we published results on the use of this device for lysis of both ex vivo RBCs and cell lines (HeLa and CHO) undergoing colloid-osmotic lysis (8). This previous work did not require automated cell recognition and tracking or detailed morphometric analysis. While automated measurements of RBC morphological parameters has been developed previously for diagnostic purposes (9,10), we extend this methodology to live cell tracking across video frames.

Here we present direct measurements of single cell morphology over the time (t = 10s) prior to the fast lysis event (t = 60ms) from microscopic data collected simultaneously for a large number of cells. Data analysis was done using an automated image analysis program performing cell recognition and tracking across video frames, along with measurements of morphological parameters. The program was entirely custom-designed and tailored to this specific application, with an extension that employs the UCSC Kestrel parallel computer to reduce the computation time to a level approaching real-time performance.

**MATERIALS AND METHODS**

**Microdevice fabrication and lytic agent generation**

A microfluidic device (Fig. 1) was designed and fabricated for local generation of hydroxide (the lytic agent). The device design and fabrication has been described in detail elsewhere (8), and was briefly as follows: Electrodes (10 nm titanium adhesion layer and 150 nm palladium layer) were photolithographically defined onto a glass slide by e-beam evaporation, followed by photoresist lift-off in acetone. Palladium was chosen as an electrode material because of its ability to electrolyze water at low voltages. The mold for the microfluidic channels and lysing chamber was fabricated using negative photoresist (SU-8 25, Microchem Corporation, 40 µm thick). PDMS (Sylgard 184, Dow Corning Corporation) was poured on the mold and cured in a 70°C oven for 6 hours. The PDMS was then carefully peeled off the mold. The fluid inlet and outlet were punched by a flat-tip needle for tube connections. The glass slide with Ti/Pd electrodes and the PDMS structures were oxygen plasma bonded.

The distance between electrodes was 600 µm, resulting in a field strength of 43 V/cm, an order of magnitude below minimum fields needed for electroporation-based
lysis (0.3 kV/cm (11), 0.9 kV/cm (12)). The chamber width and length were 1.5 mm x 5 mm. The chamber height was 40 µm.

After introducing a suspension of red blood cells (RBCs) into the chamber, a constant current was applied to the electrodes (15 µA). This insured a constant production of hydroxide at the cathode, and lead to cell membrane poration and colloid-osmotic lysis of RBCs. Erythrocytes solutions were obtained by diluting defibrinated sheep blood (HemoStat Labs, Dixon, CA) with PBS to a final hematocrit of 3.

Automated cell tracking and morphometric analysis

The purpose of image analysis was to recognize and track cells across multiple video frames, while measuring morphological parameters. We used the MATLAB (Mathworks) programming environment in order to facilitate working with images (as matrices). We performed a convolution on each pixel in the image, using a hand-picked representative cell as the kernel (inset, Fig. 2A). Pixels with high output values from the convolution were identified as positions of cells, and labeled with filled circles on the original image (Fig. 2A).

We implemented an algorithm that tracks cells across multiple frames. Since each cell moves by only a small amount in the time interval between consecutive frames, two cells in two frames with a sufficiently small variation in distance (d < 1µm) were identified as the same cell. This enabled us to automatically track cells and measure morphological parameters over time. Cells leaving the image were not counted; neither were cells that entered the image after the start of the sequence.

Once the coordinates of each cell were found, we used intensity thresholding to find the cell edge: The cell center always appears bright, surrounded by a dark ring. We used intensity thresholding to find the border between the bright central region and the outer dark ring, and defined this contour as the cell perimeter. The edge pixels were then fit with a smoothing spline, to yield a function of radius vs. central angle, R(θ). This function was numerically integrated to calculate the cell perimeter and area (Fig. 2A). It should be noted that this algorithm is critical to obtain accurate morphometric data that is not as affected by pixelization.

In order to quantify cell sphericity based on the horizontal projection of each cell, we used the circular shape factor (CSF) (13), defined by:

\[ CSF = \frac{P_{cell}^2}{4 \pi A_{cell}} \]

Perfectly circular cells will have a CSF of 1, while non-circular cells will have a CSF of less than 1. Parameters including cell position, perimeter, area, and CSF were stored in a data table for easy retrieval, data analysis, and plotting.

Computational Efficiency

The MATLAB programming environment stores variables as matrices, making it an ideal language for image processing applications. Once images are stored as matrices, thresholding and convolution operations are easily performed. However, MATLAB is an
interpreted language, meaning that MATLAB runtimes are much higher than for those of a similar compiled program.

We have developed three implementations of our automated cell analysis program: A MATLAB implementation, a C implementation, and an enhancement of the C implementation that uses the UCSC Kestrel parallel processor to speed up parts of the computation (Fig. 3, E). All implementations follow the same series of computational steps on a sequence of frames, even though the low-levels details are not identical (Fig. 3, A to D). The processing of each frame begins with a 2D convolution, after which cells positions are identified, and cells are counted. Finally, we use a simple proximity matcher to track cells across frames.

The computational times of the different implementations are reported for the processing of one frame (Fig. 3, F). We single out the time required for the bidimensional convolution because its high computational load and its regular structure motivate its implementation on a parallel architecture (the Kestrel parallel computer). The Kestrel parallel processor is a Single Instruction-Multiple Data (SIMD) parallel computer entirely designed and built at the University of California, Santa Cruz, originally targeted at computational biology applications (14,15). Kestrel is on a single PCI board for a Linux or a Windows NT host.

The bidimensional convolution is a computational step of the order of $O(N^2k^2)$, for a frame of NxN pixels and a convolution kernel of kxk pixels. The speedup we obtain performing the convolution on Kestrel is only 40%, because the Kestrel prototype has a limited bandwidth over the PCI bus that penalizes I/O-bound applications. However, our goal was to evaluate the speedup that will be possible to achieve with the new Kestrel-2 parallel processor that is being built. Kestrel-2 is based on the same architecture and technology as Kestrel, but has twice as many processing elements, runs at twice the clock speed, and has a much improved I/O bandwidth. For Kestrel-2 the convolution will take less than 20% of the total computation time. Implementing additional computational steps on Kestrel-2 or simply using a more powerful serial machine as the front-end will further improve the performance. The data in the table (Fig. 3, F) shows that the Kestrel-2 implementation will approach real-time processing, enabling additional applications such as real time sorting of cells based on morphological parameters.

RESULTS

Time course of the morphological transformations

Our method of hydroxide-driven lysis was presented previously (8), and it proceeds as follows: A DC bias (2.6 V) is applied to two palladium electrodes inside a microfluidic enclosure, resulting in the production of hydroxide ions at the cathode (Fig. 1). In the presence of hydroxide, RBCs first swell and then undergo fast radial expansion and lysis. Poration and swelling last 5 – 10s, and are followed by fast lysis, which occurs within 60 ms. Here we examine the effect of membrane poration on RBC shape before the fast lysis event. During the fast lysis event pore size is increased exponentially by osmotic pressure in a positive feedback mechanism (8).
Sequential frames were captured every 33 ms in phase contrast mode with a camera attached to an inverted microscope. Using an automated image analysis program, we were able to recognize and track cells across video frames (Fig. 2A). Cells were tracked across multiple frames, enabling the monitoring of cell shape changes over time. For each cell, the perimeter, area and circular shape factor (CSF) were calculated as a function of time (see Methods, Fig. 2A).

The survival curve (percent of the initial population lysed as a function of time) for a movie containing n = 71 cells is shown in Figure 2B. Note that hydroxide application is initiated at t = 0 s (Fig. 2B) and lysis of the cell sample is completed at t = 10 s. The average cell lifetime is $5 \pm X$ {Mr Ping please give me a number for STD} s. This curve is equivalent to survival curves customarily presented in the hemolysis literature (4,16,17). The marked difference is that our curve is quantized, with 71 distinct gradations between the first video frame to the last, where all cells have undergone lysis. Each quanta corresponds to a single lysis event. For this experiment, the cell lysis region recorded was enclosed on three sides by OH$^{-}$ generating electrodes; consequently, there was no marked dependence of cell lifetime on the distance from the electrode (data not shown, R = 0.03).

Individually, cells had a diverse range of behaviors. Data for area and CSF as a function of time are presented for three representative cells (Fig. 3). The large differences in cell data obtained were largely due to the discocyte orientation with respect to the microscope objective, for we were only presented with 2-D projections of the true 3-D cell geometry. A side view of the discocyte shows an elliptical projection that expands (Fig. 3A). The front view projection is circular and shrinks first, during the transition to a spherical morphology, followed by an increase shortly before lysis (Fig. 3B). Rotating cells give us a time-dependent oscillation between the two regimes (Fig. 3C). If averaging over a large cell sample, we expect to obtain a mean over all possible cell orientations. In order to get a better picture of cell shape leading to the lysis event, we time shifted all the morphometric parameter plots so that every cell undergoes fast lysis at the same time, t = 4.5 s (Fig. 4).

Our averaged results showed that the average CSF increased uniformly as cells approached the lysis event (Fig. 4B). None of the cells examined showed a uniform decrease in CSF, although some cells remained constant. The average CSF goes through a first period of fast increase, corresponding to the conversion of discocytes and echinocytes to spherocytes due to internal osmotic pressure (t = 0 – 2.5s), followed by another period of slow increase until the fast lysis event (t = 2.5 – 4.5s).

Cell projection area onto the horizontal plane did not increase uniformly (Fig. 4A). There was not a marked change in average area for the first 2 s of hydroxide application. This result agrees with a simple qualitative prediction: A healthy discocyte appears circular from a front view, but elliptical from a side view. As fluid entered the cell, the cell's volume would increase. Suppose the total membrane surface area remains constant. Then, as the erythrocyte shape approaches a sphere the frontal view shrinks and the side view grows in size. Only immediately prior to the lysis event (t > 4s seconds) did we observe a uniform increase in cell area. This corresponds to the cell assuming a spherical morphology, after which the pressure difference across the cell membrane causes the membrane to stretch, leading to our observed area increase prior to lysis.
Lifetime dependence on cell shape

At the start of the experiment, RBCs exhibited a continuous distribution of shapes, from discocytes to echinocytes and spherocytes. We measured lifetimes for individual cells and therefore the influence of individual cell shape on the resistance to membrane poration.

The first morphological parameter measured was circular shape factor (CSF). The minimum CSF over the first 20 movie frames was recorded, so as to be representative of initial RBC shape in the side view. A linear fit to the scatter plot of lifetime versus initial CSF shows the two to be inversely correlated ($R = 0.44$, Fig. 5B).

Another important parameter is the surface area to volume ratio (SAV) for the inflated cell. For an RBC that has reached the spherical stage, SAV is given by:

$$\frac{A_{\text{surface}}}{V_{\text{final}}} = \frac{4\pi R^3}{4\pi R^3} = \frac{3}{R}$$

Because poration is a surface phenomenon, we expect lifetime to be inversely correlated with the surface area to volume ratio in the spherical stage (before the fast lysis event). Figure 5C shows that the two are indeed inversely correlated ($R = 0.43$), about as strongly as lifetime and initial CSF ($R = 0.44$). An important internal check is to verify that initial CSF does not correlate with spherical SAV. The data shows no correlation, with a linear regression coefficient of $R = 0.01$ (data not shown). In Figure 5D, we plot lifetime as a function of the inflation volume over cell surface area, $(V_{\text{final}} - V_{\text{initial}})/A_{\text{surface}}$. This measure shows the best correlation to cell lifetime ($R = 0.65$).

**DISCUSSION**

Hydroxide is known to react with cell membranes, cleaving constituent molecules into fatty acids and lysophospholipids, a process known as saponification (18). As the nomenclature suggests, lysophospholipids have been shown to porate cell membranes and lead to lysis when added to extracellular media (19). Therefore, hydroxide application is likely to lead to lysophospholipid mediated poration of the cell membrane. The pores allow small ions to permeate the membrane while keeping larger anionic proteins in the cytoplasm. Small ions and associated water molecules enter the cell, creating a positive osmotic pressure. The swelling and subsequent bursting of the cell, referred to as colloid-osmotic lysis (4), was shown to be a conserved mechanism for a large number of pore-forming compounds, from bacterial toxins to viral capsids (1).

We measured morphological parameters for single cells undergoing this process with two aims in mind: First, to quantify morphological changes prior to fast lysis, and second, to determine the influence of initial cell shape on resistance to porating agents. Automated analysis of RBC morphology from single images has been reported previously, with an emphasis on the diagnosis of sickle cell anemia (9,10,20). These measurements were taken on fixed cells between microscope slides, while we measure live cells in a fluidic enclosure. In addition to developing our own method for measuring morphometric parameters, we implement cell tracking across multiple frames, enabling
the monitoring of cell shape changes ex vivo over time. The analysis of program run times suggests that real time processing of the image data will be possible by using a parallel accelerator, such as the UCSC Kestrel parallel computer (see Methods).

Morphometric parameters were averaged over the cell population after offsetting the time scale such that all cells undergo fast lysis simultaneously, at \( t = 4.5 \) s. The average CSF (sphericity) goes through a period of fast increase \((t = 0 – 2.5s)\), after which it remains constant until the lysis event \((t = 2.5 – 4.5s)\). This result suggests a relation between the pressure required to overcome the RBC shape conserving factors and the pressure required to overcome the membrane tensile strength. An exact relation would require knowledge of the rate of increase in internal osmotic pressure as a function of time, in addition to the present data on cell shape. If we make the simple assumption that pressure increases at a constant rate, morphometric data shows that shape conserving factors are overcome by half the pressure required to overcome membrane tensile strength. Erythrocyte shape is believed to be governed by a combination of bilayer coupling and the membrane-associated cytoskeleton (21). In the future, measurements of cell morphology changes in the presence of either cytoskeleton disruptive agents or membrane coupling alteration compounds can be used to discriminate between the two shape control mechanisms.

The second parameter measured was cell area, or rather the cell’s projection onto the horizontal plane. As described above, for the first half of the cell lifetime discocytes convert to spherocytes due to increased internal osmotic pressure and swelling. This process can lead to either increases or decreases of cell area, depending on the discocyte orientation with respect to the horizontal. This result is illustrated well by the sample cell in Fig. 3C: Since the cell is rotating constantly, the peaks in the area graph correspond to a frontal view, while the troughs occur for side views. Note that during the transition to a sphere \((t = 0 – 5s)\) the side view area increases and the frontal view area decreases, but the average area remains approximately constant until the last second \((t = 6 – 7s)\), when there is a monotonic increase. One could simulate the predicted change in projection area shape and size as a function of flux with the assumption of a constant surface area for the cell. However, without determining the discocyte orientation, the model predictions would be impossible to check. The uncertainty in the cell orientation constitutes the most serious impediment to extracting the 3D cell shape from our 2D data. However, since the orientation angle is likely to be random, averages over a large number of individual cells will exhibit the correct behavior.

The influence of individual cell shape on the resistance to membrane poration can be elucidated by correlating cell lifetimes to morphological parameters. At the start of the experiment, RBCs exhibited a continuous distribution of shapes, from discocytes and echinocytes to spherocytes. Our data shows that sphericity (CSF) is inversely correlated to cell lifetime (Fig. 5B, \( R = 0.44 \)). This relationship is present despite the measurement noise introduced by the distribution in RBC orientation, which makes some discocytes appear spherical. Therefore, a larger scatter in lifetimes is observed for cells that appear spherical (CSF > 0.96). This relationship is consistent with a constant flux model for colloid-osmotic lysis: Discocytes start out with a smaller ratio of volume to surface area; therefore, for a constant inward flux per membrane area, the transition to spherocytes causes a delay in the application of osmotic pressure to the taut spherocyte membrane. This relationship agrees well with data showing that spherocytic RBCs have a greater
osmotic fragility (3). In spherocytosis, a patient's RBCs assume a spherical shape due to defects in RBC membrane associated cytoskeletal proteins (22,23). However, osmotic fragility measurements on spherocytotic cells leave open the possibility that osmotic fragility is not due to cell shape alone, but to differences between the cytoskeletal makeup of spherocytotic and normal RBCs. In this context, our data shows that normal RBCs that are spherical also display a greater osmotic fragility. This is an important result, showing that morphology alone can be responsible for increased osmotic fragility.

Membrane poration is a surface phenomenon, while osmotic pressure buildup due to flux across the membrane is dependent on the fractional increase in total cell volume. Thus cell lifetimes should depend on any variation of volume to surface area, which is dependent initial cell shape (as described above), but also on total cell size in the spherical stage. Spherical cell size was determined by measuring the cell projection area in the final stages before lysis, when there is no dependence on cell orientation. The spherical cell radius is inversely correlated to the final surface area to volume ratio (defined as $SA_2/V_2$ in Fig. 5A). The final surface area to volume ration is indeed inversely correlated to lifetimes also, while uncorrelated to the initial CSFm (Fig. 5C, $R = 0.43$). If we assume a constant flux per unit area into the cell, lifetime should vary with the ratio between the inflation volume and the surface area at the spherical stage. The total inflation volume over the duration of the experiment can be calculated by subtracting the initial volume ($V_1$, in the discocyte state) from the final volume ($V_2$, in the spherocyte state). Figure 5D shows the best correlation to cell lifetimes is found by looking at the inflation volume to surface area ratio, a metric that includes both measurements on the initial cell shape and the final cell size. We therefore conclude that osmotic fragility is higher for cells that are initially more spherical or smaller than average.

Our lifetime measurements for RBCs show that cell size is correlated with cell lifetime under osmotic stress. This observation suggests the existence of an even stronger variability of osmotic fragility between different cell types, which differ greatly in size and therefore volume to surface area ratio. In previous experiments (8), we have shown that larger HeLa and CHO cells ($R_{cell} = 12-17 \mu m$) display a longer duration of the fast lysis event. The duration was found to increase with the volume to surface area ratio, from 60 ms for RBCs to $159 \pm 64$ ms for HeLa cells (8). Therefore, the dependence on cell size holds for the time scale of lysis event, despite the differences in cytoskeletal structure between the different cell types.

In conclusion, we present data on morphological parameter changes for RBCs in the presence of hydroxide, a membrane porating agent. Measurements of individual cell data were performed using an image analysis program for automated cell tracking and morphometric measurements designed and implemented in our laboratory. The most important result is the dependence of resistance to porating agents (and osmotic fragility) of individual cells on their inflation volume to surface area ratio. The ratio is dependent on both initial cell shape (discocyte vs. spherocyte) and cell size (cell radius in the spherical phase). This relation shows that a constant flux per unit area model fits the data on poration based RBC lysis. The higher osmotic fragility associated with the blood disorder spherocytosis, which exhibits spherical RBC shapes, is explained by this model. The methods outlined here may be used in future studies of red blood cell morphology changes in response to physiological toxins.
Figure Captions

**Figure 1** Device design for local hydroxide generation. A voltage bias is applied to palladium electrodes inside a microfluidic chamber, delivering hydroxide ions to RBCs in suspension in the vicinity of the cathode. The microfluidic chamber consists of a PDMS channel bonded to a glass slide containing microfabricated electrodes. Changes in cell morphology are recorded by microscopic observation of cells undergoing lysis in the vicinity of the cathode.
Figure 2  Cell recognition and percent lysis over time.  (A) A typical frame after cell recognition and edge detection. Cells are marked by colored spots and labeled by ID numbers. The sample cell used for convoluting the image is shown in the upper left corner. The color of the spots corresponds to the quality of the match, ranging from red (90-100% of maximum convolution output) to blue (50-60% of maximum). Individual cells in consecutive frames are matched by a simple proximity tracking method. Cell edges were found by intensity thresholding and labeled with the green contour. (B) Percent lysis as a function of time for a frame sequence following the image in A (dotted line). {Mr Ping please change the graph in B to have a solid blue line for the fit – I’ll add it to the fig. for B&W clarity}. A Gaussian distribution of lysis events corresponds well to the sigmoidal shape of the survival curve (solid curve).
Figure 3 Sample frames (details) from the main computational steps in our programs, and comparative performance of our implementations. All times are for 512x512-pixel frames with 8 bits per pixel and a convolution kernel of 15x15 pixels, on a 1 GHz Pentium III. The UCSC Kestrel parallel accelerator (E) and Kestrel-2 (currently in its final design stage – performance is projected), only implement the convolution, and interface with the C pre- and post-processor.
Figure 3  Morphological analysis of three representative cells prior to lysis. Circular shape factor (solid line) and cell projection area (dotted line) are plotted with respect to time (note the different scales). (A) Side view of a cell at constant orientation. Both area and CSF are monotonically increasing. (B) Frontal view of a cell at constant orientation. This cell goes through much smaller changes in size and CSF. However, we do notice that its size is slightly declining. These results agree with a front view of a discoid shape that is slowly converted to a sphere. (C) This cell is simultaneously flipping over and changing to a spherical shape. This results in an oscillating CSF that increases over time. It also causes an increase in size of the side projection and a decrease of the frontal projection, so that the area oscillation amplitude decreases.
Figure 4  Average cell area and CSF over the cell population as a function of time. (A) Cell projection area change as a function of time. (B) Circular shape factor change as a function of time. The timescales for each individual cell were normalized to a common time of lysis (4.5 s) before taking the mean. Error bars represent 1/2 of one standard deviation from the mean both above and below the graph. The large standard deviation in the data is caused by the diversity of the cell shape and orientation across the population.
Figure 5  Cell lifetime dependence on morphometric parameters.  (A) Cartoon representation of the changes in cell shape from initial discocyte (volume $V_1$, surface area $SA_1$) to the final spherical shape before lysis ($V_2$, $SA_2$).  The scatter plots relate lifetime to initial CSF (B), final spherical surface area to volume ratio (C), and the inflation volume to surface area ratio (D).  The initial CSF was taken as the minimum over the first 20 frames (0.75 s) of cell lifetime, in order to account for cell rotation.  The final surface area to volume ratio (C) was averaged over the last 0.5 seconds before lysis.  Lifetime is inversely correlated with both initial CSF ($R = 0.44$) and final surface area to volume ratio ($R = 0.43$).  The best correlation is found between lifetime and the inflation volume to surface area ratio (D), in agreement with a constant flux per unit area model.

Acknowledgements: The authors wish to thank Nima Aghdam for 3D graphic design. This work was supported in part by the NSF grant EIA-9722730 (A.D.B. and R.H.).

LITERATURE CITED


