Chemical Analysis of Single Human Erythrocytes

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Scientists have long been fascinated by the elementary entities that make up matter. We have seen the natural progression from molecules to atoms to nuclei to quarks. In living systems, the analogy is the series from animals to organs to cells to subcellular organelles. As the study of biophysics and biochemistry becomes more sophisticated, there is a growing need for monitoring biological events at the cellular and subcellular levels.

The study of the chemical contents of individual mammalian cells presents a unique challenge. A human erythrocyte is roughly 7 μm in diameter and has a volume of 90 fL. The sheer smallness of such a "sample" makes many traditional analytical methodologies inapplicable. What is important here is the *mass* limit of detection (LOD) of a method, that is, the smallest amount of an analyte that can be detected. This is in contrast to the more common measure of performance, the *concentration* LOD. This is because, in most other practical applications, one has a relatively large volume of sample, so that signal averaging over a long measurement time, preconcentration, or chemical derivatization of the analyte before measurement can be implemented.

Although it is now possible to count individual atoms[1] and molecules,[2–5] the situation is a bit more complicated in dealing with single cells. The complex mixture of components that are present very likely will interfere with the measurement process. What can be achieved in terms of LODs for neat samples is almost never transferable to real samples.

Chemical analysis of single cells has been achieved through a variety of techniques. The separation and direct observation of several hemoglobins was reported almost 30 years ago.[6] Flow cytometry is a well-established tool in cell biology. Fluorescence microscopy,[7] especially with the recent development of confocal imaging,[8] is another broadly applied technique. The detection of individual ions has been demonstrated in patch-clamp experiments.[9] The challenge remains to make single-cell analysis more general, more sensitive, more selective, more quantitative, and more informative (by allowing simultaneous determinations of many analytes).

A general approach to characterize complex samples is to incorporate chemical separation before measurement. In capillary liquid chromatography (LC),[10] the internal diameter can be as small as 2–5 μm. Individual cells injected will not suffer unacceptable dilution during separation. Another recent development is capillary electrophoresis (CE),[11] which provides even better separation efficiencies because of a flat flow profile and the lack of slow mass-transfer kinetics. Noteworthy is that CE is typically performed in a water-based medium and is inherently friendly to biological molecules.

Detection of the separated components, however, remains problematic, since most compounds of biological interest do not possess the requisite chemical or physical properties that are amenable to sensitive detection. For large cells such as snail neurons, the sample volumes are around 1 nL. Miniaturized versions of thin-layer chromatography,[12] mass spectrometry,[13] LC,[14] and enzymatic radiolabeling have been successfully applied. An example of micromanipulation and sensitive voltammetric detection after separation involves the analysis of single snail neurons.[15] A version of this[16] has been applied to the characterization of individual bovine adrenal medullary cells (1–2-pL volume), with LODs in the femtomole range for electroactive species. CE has also been used to analyze snail neurons, by a microinjector to sample the intracellular fluid.[17]

A legitimate question is why single-cell studies are interesting. There is the basic issue of homogeneity within a population. Are all cells serving the same

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Table 1. Representative Analytes in Human Erythrocytes

species	amount/cell	function	related enzymes
K, Na	8, 1 fmol	ionic balance	Na,K-ATPase
lactate, pyruvate	1.3, 0.8 fmol	metabolism	LDH
glutathione (GSH)	0.2 fmol	antioxidant	glutathione reductase, G6PDH
hemoglobin A_0	450 amol	oxygen transport	methemoglobin reductase
carbonic anhydrase	7 amol	CO_2 transport	self
lactate dehydrogenase (LDH-1)	30 zmol	metabolism, cancer	self
glucose-6-phosphate dehydrogenase (G6PDH)	20 zmol	hexose shunt, GSH, cancer	self

simple function (e.g., erythrocytes) alike? If not, why not? How are the physical characteristics of cells related to their chemical compositions? What are the cooperative effects in groups of cells versus isolated cells? Single-cell insights may also benefit clinical diagnosis and treatment. In regular blood tests, hundreds of thousands of cells are homogenized to provide sufficient amounts of analytes for quantification. At the early stages of disease or carcinogenesis, only a few cells may carry the specific chemical or biochemical markers indicative of infection. Such markers are likely to be completely masked by the averaged contents of the overabundant healthy cells. On the other hand, if cells are examined individually, the chances of recognizing abnormal cells are substantially better. In the treatment of diseases, it is also likely that the uptake of pharmaceuticals can be very different between healthy and disease-stricken cells. The understanding of such variability can lead to better drug design and control of side effects. Cells also represent a fundamental entity that serves as a model for developing biomimetic systems and nanostructures.

In this Account, we review some of the recent work in our own laboratory in the area of single-cell analysis. The human erythrocyte was selected for study since the existence of extensive information on it[18] allows for comparisons and evaluations of the techniques. The small size implies that any techniques developed will likely be applicable to other mammalian cells as well. The main tool employed is the laser, which is known to provide sensitive spectroscopic measurements in small samples with outstanding LODs.[19] Optical monitoring of microscale separation systems also avoids specialized interfaces[20] and possible degradation of the separation process.[21]

Overall Approach

There are hundreds of interesting species even in a cell of restricted function such as erythrocytes. In Table 1 we list some of the typical components that are among the most important in terms of cell function and viability. Also included are examples of specific functions served by these components and the approximate amounts of each present. The challenge is to establish methods that have the required sensitivity and yet provide enough discrimination from other species that are present at their native levels. Capillary electrophoresis clearly has the high separation efficiency to deal with very complex mixtures.[11] It should be straightforward to separate the major cations and anions from each other and from the proteins.

To take advantage of the microscale CE system, the cells must be transferred directly into the capillary column. This can be accomplished under the field of view of an optical microscope.[22] A sample droplet containing many blood cells is placed on a standard microscope slide. The injection end of the separation capillary can be brought into the same observation region with the aid of micromanipulators. In practice, if the capillary tip is first etched down in size with hydrofluoric acid,[23] movement of the capillary will cause minimal disturbance in the sample droplet. The capillary opening can then be aligned with the selected erythrocyte. By pulling a vacuum through an airtight syringe connected to the opposite end of the capillary, the selected erythrocyte can be drawn into the column.

Once injected, the cells tend to adsorb onto the capillary walls due to hydrophobic interactions and become immobile. The extra matrix fluid that was injected with the cell can be pushed back out to avoid interference with the separation or the detection. The capillary can then be removed from the micromanipulator and placed in the vial containing the running buffer. When high voltage is applied, the buffer solution is drawn over the immobilized cell. By using running buffers at <20 mM ionic strength, lysing is achieved within 1−2 s by osmotic pressure. The intracellular fluid is completely released, completing the sample injection process.

A few subtle points concerning the use of CE in single-cell studies should be mentioned. Because of the complex sample matrix, it is likely that the nature of the capillary walls will change as successive cells are injected. This can alter the electroosmotic flow rate and in turn the migration times of the components. Fortunately, the total amount of material injected, even over a series of 50 erythrocytes, is still small compared to the nanoliter volumes typically used in CE. We have confirmed that migration times vary by less than 20% in such cases, with relative migration times changing in the ±3% range.[23,24] So, column reconditioning is generally not needed in between cell injections. Analyte identification is based on migration times compared to standard samples. Because direct confirmation by, e.g., mass spectrometry is not possible at these low amounts, we rely on the known average compositions of these cells to provide additional confidence in the peak assignments. By interposing cell injections with runs of standard solutions, we estimate that the quantitative precision in most cases is around ±5%. Finally, contamination is a serious issue at these low analyte levels. The high

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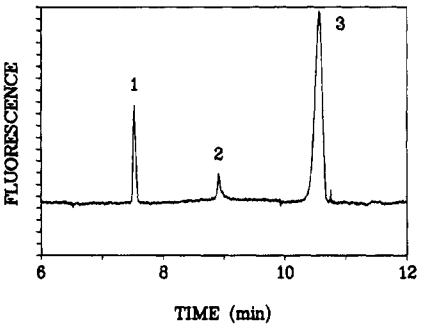

Figure 1. Electropherogram of mBBr-derivatized contents of a single human erythrocyte. Peak 1 = unreacted mBBr; peak 2 = unidentified intracellular thiol; peak 3 = glutathione (~100 amol).

separation efficiency of CE solves most of these problems. The only exception is the determination of Na and K. We estimate that a 2-μm particle (e.g., dust) will release 1–2 fmol of Na and K due to surface adsorbed materials.[25,26]

In Vivo Fluorescence Derivatization

To take advantage of the impressive LOD offered by laser-excited fluorescence, one needs highly efficient fluorophors. Since most naturally occurring analytes are not good fluorophors, chemical derivatization (tagging) is a viable approach. Cell biologists have already established an arsenal of fluorogenic reagents for staining specific analytes. Reduced glutathione can be derivatized *in vivo* by monobromobimane.[27] In effect, one is using the cell itself as the reaction vessel. This is critical because any attempt to harvest the cell, lyse it, and introduce the fluorogenic reagent in an external vessel will unavoidably lead to dilution and thus loss in sensitivity.

Figure 1 shows an electropherogram of the intracellular fluid of a single erythrocyte after it was treated with monobromobimane,[22] confirming the high performance of fluorescence detection in these small volumes. Figure 1 shows an important difference between fluorescence microscopy and CE, even though both involve the identical cell preparation procedure. The signal from all three components will contribute additively in fluorescence microscopy. CE on the other hand allows reliable quantitation of the fluorescent product even though excess reagent may be present. Separation is also important when more than one analyte binds with the fluorogenic reagent, as would be expected in complex samples. In general, LODs around 1 amol for single-cell studies can be achieved.

To show that one can follow intracellular biochemical events and to verify the identity of the peaks in the electropherograms, we demonstrated that peak 3 in Figure 1 disappeared on treatment with diamide[28] and reappeared when dithiothreitol[29] was added. These reagents serve to oxidize and then re-reduce glutathione. A 7-fold cell-to-cell variation in glutathione content was observed. The distribution within the cell population was similar before and after the modulation experiments. One can conclude that the cell-to-cell variation is not due to partial oxidation of intracellular glutathione during sample handling and subsequent chemical derivatization, but represents actual differences within the cell population.

There are some limitations on the use of *in vivo* derivatization for single-cell studies. Since an incubation period is needed, one cannot guarantee that the cell is in its native state when analysis is performed. The derivatization process inherently changes the distribution of the intracellular components, and the living entity may react to this change by compensating for the consumed analyte. The selectivity of the fluorogenic reagent is another concern because of the presence of many related species in the cell. The exception is if an immunospecific reagent or sequence-specific reagent is used.

Indirect Fluorescence Detection

Indirect detection is used in CE for the determination of both organic and inorganic compounds that do not possess a suitable detection property.[30] The buffer ion is selected to produce a large signal at the detector. Due to charge displacement, a lower signal is then observed in the analyte zone to produce a negative peak. The LOD for indirect fluorescence detection is on the order of 10^{-7} M or 5×10^{-17} mol of analyte injected.[31] Although conductivity[32] and potentiometric[33] detection can in principle be used for ionic analytes, these low-mass LODs have not yet been demonstrated in CE separations. In fact, the low sensitivity compared to fluorescence detection allows simple electropherograms to be obtained, as all other ionic species are generally present at substantially lower concentrations in cells.

Indirect fluorescence detection is experimentally more demanding compared to fluorescence detection. A small change on top of a large background signal is monitored. In addition to a stable excitation intensity, the concentration of the background fluorophor must also be stable. In the study of single cells, the intracellular fluid, which is mainly water, can lead to dilution peaks in the electropherogram. The matrix material can also adsorb and desorb from the column walls, disturbing the equilibrated fluorophor concentration and producing base-line drifts. The problem is magnified when a large amount of the cell-suspension liquid is also drawn into the capillary. For the case of red blood cells, the use of a suspension fluid of 4–8% glucose and a running buffer containing 1% glucose allows cells to be preserved prior to analysis without creating unacceptable base-line disturbances on cell injection.

In the early stages of this study,[22] the reproducibility for determining K and Na in single erythrocytes was very poor. This was later found to be due to the contamination of K and Na from the environment.[34,35]

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Also, intracellular K and Na levels would decrease over long times or during low-temperature storage of a blood sample. Once the proper experimental protocol was established, Na and K amounts in individual erythrocytes could be quantified with confidence.[34] In a series of 49 cells, a large cell-to-cell variation in the K content was observed. Similar results were obtained in a study of 27 erythrocytes when intracellular lactate and pyruvate were monitored.[23] A subtle feature in indirect fluorescence detection is that laser power changes, optical alignment problems, and detector response variations are constantly recorded as the background fluorescence level. Our experiments clearly show that such fluctuations are not the major cause of the observed signal variations. Rather, there are inherent differences among erythrocytes in a given population.

Native Protein Fluorescence

One approach to sensitive detection of proteins is to introduce fluorescence tags onto the molecule. A complication arises because proteins in general contain multiple sites for derivatization. A single protein can thus produce a host of products, each with a different number of fluorescent tags. Because of this, CE separation of precolumn derivatized proteins results in broad peaks, degrading resolution, and quantitative reliability. Proteins can in principle be detected by indirect fluorescence detection.[36] However, even the most abundant proteins in erythrocytes are below the present capabilities of indirect fluorescence detection.[33]

Proteins are actually weakly fluorescent molecules as a result of the aromatic amino acids. The main point is that laser-excited fluorescence is such a sensitive detection mode that even if one gives away several orders of magnitude in performance due to the low fluorescence quantum yields, the LOD is still within a useful range for studying the major intracellular proteins. Continuous-wave laser emission at 275 nm is available and is preferable to pulsed-laser systems because of the better focusing properties and because damage to the capillary columns is not a problem. Fused silica capillaries tend to fluoresce when excited in the deep UV, presumably due to the presence of rare-earth inclusions. Even distilled and deionized water contributes to background fluorescence, since trace organic contaminants are always present. However, by selecting the appropriate pH to maximize the fluorescence efficiency and to avoid band broadening, one can achieve LODs in the 10^{-10} M or 10^{-19}-mol range.[37]

Separation of proteins presents another challenge. The surfaces of the fused-silica capillary form adsorption sites for proteins. Many surface-treatment procedures have been suggested for protein separations,[38] but there is no universal solution. The situation is simplified when one is dealing only with the major proteins in erythrocytes. We have shown[39] that the injection of 1 amol of a model protein results in a fluorescence peak that represents 90–95% recovery of the injected material. Application of native protein fluorescence to single-cell studies is therefore feasible.

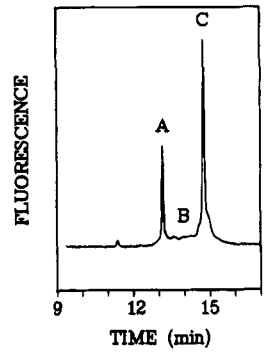

Figure 2. Electropherogram of major proteins in a single erythrocyte. Peaks A, B, and C are carbonic anhydrase (~7 amol), methemoglobin (~5 amol), and hemoglobin A_0 (~450 amol), as identified from migration times relative to standards.

A representative electropherogram of the proteins in a single erythrocyte is shown in Figure 2.[24] The major proteins, hemoglobin A_0, methemoglobin, and carbonic anhydrase, are clearly depicted. We found that there were large variations from cell to cell in the amounts and the relative abundances of each protein. Since these cells have been stored for up to 3 days, oxidation of hemoglobin to methemoglobin explains most of these variations. The rate of change on storage is apparently not uniform within the cell population.

Fluorescence Enzyme Assay

As one goes below the 1-amol level, the number of intracellular components that are present increases dramatically. Even if techniques such as native protein fluorescence can be further developed to achieve sub-attomole LODs, separation of these components to produce interpretable electropherograms may become an intractable problem. It would be important to further increase detection selectivity while pushing sensitivity.

It has already been shown that 10^{-17} mol of an enzyme can be detected in a miniaturized version of standard enzyme assays in a capillary format.[40,41] The adaptation to fluorescence-based detection further lowers the LOD to the 10^{-21}-mol range.[42,43] Briefly, enzymes can catalyze the turnover of substrates to products to provide large amplification factors for detection. LDH *in vivo* converts pyruvate and NADH (nicotinamide adenine dinucleotide, reduced form) to lactate and NAD^+ in an important link in the metabolic cycle. To achieve favorable detection, we forced the reaction to go in the reverse direction, which is also catalyzed by LDH. This way, the substrates do not contribute significantly to background fluorescence.

In an actual experiment, high voltage was applied to the capillary to allow LDH to mix with the substrates due to differential mobility. At the same time, the various isoenzyme forms of LDH were separated

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due to zone electrophoresis. To magnify the signals further and to provide resolution among the isoenzyme forms, electrophoresis was interrupted after 1 min and incubation was allowed to take place for 2 min. During incubation, distinct zones of products are created where the enzyme forms reside. This leads to chromatographic-like peaks that depend only on the enzyme activity and the incubation time. Our single-cell studies[42] show that the major LDH isoenzyme forms can be reliably quantified.

Detection by fluorescence enzyme assay was demonstrated for LDH-1 down to 10^{-21} mol (800 molecules). It should also be possible to introduce multiple sets of substrates to assay several enzymes simultaneously. The enzymes can be separated electrophoretically before incubation, the products can be separated electrophoretically after incubation, or the products can be discriminated spectroscopically. Since one can in principle design suitable substrates to key on specific enzymes to implement a fluorescence enzyme assay, this technique appears to be broadly applicable for studying the minor intracellular components.

Particle Agglutination Immunoassay

There are, however, still many other important species that are present at sub–attomole levels that cannot be detected by any of the above techniques. We recently developed a miniaturized version of a particle-counting immunoassay to address this issue.[44] Antibody-coated latex particles are used as the reagent. When they are incorporated into the electrophoretic buffer and flow past a laser beam, spikes due to light scattering from individual particles can be observed with a photomultiplier tube, provided that the particle concentration, the capillary diameter, and the laser beam size are optimized. When an antigen is present, agglutination takes place to form larger particles. An appropriate discriminator–counter system can then be used to monitor the large scattering spikes due to the antibody–antigen reaction.

In principle, single antigen events can be recorded. In practice, some background counts due to particulate inclusions in the buffer solution exist, even after ultrafiltration. The laser intensity is also not uniform across the capillary, so that particles at the center produce different scattering intensities compared to those near the walls. The tumbling motion of the asymmetric agglutinated particles also causes variations in the signal. So, selection of the discrimination threshold is not well defined but must be optimized. Even if perfect discrimination can somehow be achieved, incomplete reaction still limits the signal. Diffusion of the particles would also limit the production rate of the dimers. In the end, we were able to detect glucose-6-phosphate dehydrogenase (G6PDH) at 10^{-21} mol (600 molecules) using this scheme.[44]

The distribution of intracellular G6PDH in a population shows large cell-to-cell variations that have been seen before in cell fractions[45] and have been attributed to age variations of the red cells. A subtle point is that immunoassays reveal the amounts of material present while enzyme activities relate to the functional forms only. Even if the enzyme becomes inactive due to conformational changes or chain modifications, the antibody may still recognize certain specific regions to produce a response. The information is thus complementary. It should be possible to eventually perform both types of assays on the same cell, presumably for any enzyme, since the two signals are easily distinguishable spectroscopically.

Laser-Generated Microplasma Emission

CE is not the only approach that allows the simultaneous measurement of intracellular components. Recent developments in laser-generated microplasmas have led to femtomole LODs for elemental species *via* the associated atomic emission.[46] The idea is that blood cells can be lined up in a narrow flow stream to intersect a high-power laser beam. Absorption of the laser light leads to intense local heating and the eventual production of a microplasma. Proper collection of the atomic emission through interference filters allows several elements to be quantified at the same time.

Several design features were found to be important in these experiments.[47] In the standard sheath-flow arrangement,[2] the sheath liquid represents bulk that needs to be vaporized and heated at the same time. This seriously quenched the plasma, and no emission was observed. However, by modifying the sheath flow so that the core flow of cells rides on the *outside* of the sheath liquid, a hot plasma can once again be generated. Also, the LOD was sufficient for quantifying intracellular K in a single cell, but was marginal for quantifying intracellular Na. This was overcome by deliberately flowing the cells past the laser beam more than one at a time. This increases the signal levels for confident measurements. Even though the experiments are not on one cell at a time, distribution of K and Na in the cell population can still be obtained on the basis of Poisson statistics.

Implications

In all of the studies described above, we found without exception that there are large cell-to-cell variations for each analyte. This cannot be explained simply as variations in cell volume, as healthy human erythrocytes are known to change by less than ±10% in volume.[18] Particularly noteworthy is that independent measurements of intracellular K and Na by CE[34] and by atomic emission[47] yielded nearly identical distributions within a cell population. The major source of inhomogeneity in erythrocytes in an individual is the cell age. Erythrocytes are unusual in that they are not nucleated and lack the ability to replenish or repair proteins. As these circulate in the blood, they are exposed to various damage pathways and gradually degrade over their 120-day lifetime. So, one expects that younger cells contain larger amounts of proteins[24] and higher enzyme activities.[42] From Table 1, it is clear that all the species studied are related to regulating enzymes or are enzymes themselves. It is not surprising that each analyte exhibits similar behavior. Additional clues come from the fact that the amounts of certain pairs of analytes are

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correlated in single cells, for example, hemoglobin A_0 and carbonic anhydrase,[24] and LDH-1 and LDH-2.[42] Degradation over an identical period of time would cause the amounts of such pairs to decrease simultaneously.

Ideally, one needs an independent age marker to confirm these correlations. Cell density has been related to cell age, but the correlation is not unambiguous.[48] Transfusion experiments can provide definitive results, but these are practical only in animals and not in humans. One possibility is to use the degree of glycation of hemoglobin[49] as an age marker. This is a direct result of the cumulative exposure of erythrocytes to blood glucose over their lifetime. If a procedure can be worked out to separate out this specific form of hemoglobin in single cells, the above hypothesis can be rigorously tested. It appears that simultaneous determination of several intracellular components in the same cell is the key to many important biological questions.

The clinical value of single-cell analysis is promising. It has already been shown that LDH-4 and LDH-5 are present at elevated levels in various diseases, including colon cancer, breast cancer, liver cancer, and leukemia.[50] The feasibility of reliable measurement is enhanced by the fact that only ratios of the isoenzymes will be evaluated. It would be interesting to take a biopsy sample and to study such ratios as a function of the progression from normal tissue to benign tumor to cancerous tissue, cell by cell. The question is if chemical changes precede physical changes in such situations, in which case diagnosis becomes possible before pathological identification is possible.

If such measurements show promise for clinical diagnosis, one then needs to address the question of speed and cost. For the CE-related procedures, separation times and incubation times will limit each determination to 10−15 min. However, capillaries can be run and monitored simultaneously in one instrument to greatly increase the processing speed. Our recent work has led to the demonstration of running 100 electropherograms simultaneously,[51] with a projected performance of 4000 capillaries in one instrument. Flow cytometry can presumably be interfaced to such a system, so that cell injection can also be automated.

As analytical technology advances, so will more questions in biology become tractable. Single-cell analysis has opened up a new level of detail for relating chemical contents to biological function. The next few years should be exciting ones for both the development and application of single-cell analytical schemes.

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