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A Scalable Cell-Loading System for Non-Viral Gene Delivery and Other Applications

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Across many areas of biopharmaceutical development, the goal of consistently transfecting appropriate quantities of DNA into cells has often been a significant bottleneck. Electroporation — a method of temporarily permeabilizing cell membranes by using a short electric pulse — has gained ground in recent years as an effective means of transfection. A cell loading system based on electroporation has been designed for *ex vivo* cell modification in a clinical setting and for incorporation into cGMP processing applications. In the MaxCyte system, cells are suspended in buffer containing the biomolecule to be loaded are passed through the processing chamber, and then are available for further operations, cryopreservation, or administration. The system is scalable, employs a sterile, closed, disposable processing unit, and no chemical or biological substances are added.[1,2]

The computer-controlled MaxCyte system (Fig. 1) consists of a power/control assembly approximately the size of a personal computer, and a disposable processing unit that attaches to the assembly. The materials used in the components have been selected for biocompatibility under the conditions of electroporation.

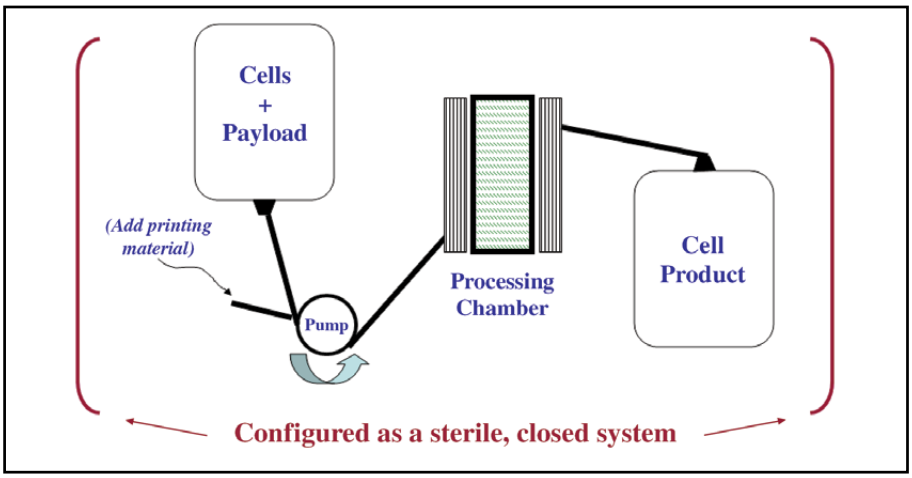

Figure 1. Schematic of the MaxCyte System. A cell suspension containing the biomolecule to be loaded ("payload") is pumped through the processing chamber where an electric field is applied synchronously with the flow rate. The loaded cells are collected in an appropriate container. Additional containers for priming fluid(s) and waste fluids can be incorporated into the sterile, closed, disposable processing unit.

Introduction

All cells are bounded by an outer plasma membrane that maintains a selective surface barrier to the entry of molecules that is essential to cellular integrity and viability. Many bioactive molecules that could potentially modify cellular activity and bring therapeutic benefit do not cross this barrier. Although molecules can be specially designed or selected that cross this barrier, many small molecules, particularly highly charged ones, and nearly all macromolecules, including nucleic acids, do not efficiently enter cells under normal conditions.[3]

Several approaches have been developed to allow the entry of molecules, particularly DNA into cells: packaging a genetically engineered gene construct into a virus or virus-like particle use of lipid vesicles that encapsulate or coat DNA and are taken up by the cell or fuse with the plasma membrane; and electroporation. The latter was developed based on the observation that brief electric fields result in the formation of transient permeability of the plasma membrane through which molecules, including DNA, can enter the cell by passive diffusion or electrophoretic transfer. If properly applied, the permeability resolves and cells rapidly return to a basal state after application of the electric field. The MaxCyte System is based on electroporation and has the distinct advantage of not requiring the inclusion of a secondary agent, (e.g. a lipid, viral package or carrier protein) which can be immunogenic or toxic. The only mechanism used is an electric field.

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The Process of Electroporation

Although electroporation is a technology that has been routinely used in research laboratories throughout the world for the past 30 years and has been adequately reviewed, some general comments on the process are appropriate. The primary application has been in delivering genetic constructs into eukaryotic and prokaryotic cells, resulting in non-viral transfection of such cells. The process subjects cells to a pulsed electric field for a short duration, resulting in permeabilization of the lipid bilayer of the cell membrane. This permeability develops in microseconds and resolves in seconds to minutes. While a physical "pore" has been observed under some circumstances, in most situations the permeability change is probably related to transient reorientation of membrane phospholipids. During the permeable period, both polar and nonpolar molecules of various sizes can diffuse through the permeable areas according to concentration gradients. In addition, the electric field provides a force by which charged particles move into the cell ("electrophoretic" mechanism). Various molecules can be inserted into cells, including drugs, DNA, proteins, or other biomolecules.

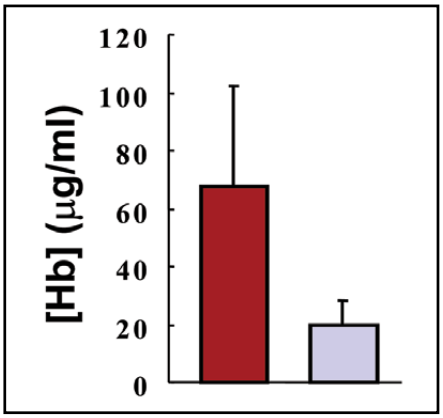

Figure 2. Approximately 70% less hemoglobin was detected in the Matrigel from mice injected with mIL-12 transfected cells (right column) than the Matrigel from control mice (left column).

The MaxCyte System

The cell loading system described in this report, while based on the physical process of electroporation, differs from instruments that have been available for laboratory use in a number of ways:

- Existing electroporation technology is limited by its ability to treat only small suspensions of cells (usually < mL). MaxCyte utilizes a computer-controlled flow system that synchronizes application of electric field to the flowing cell stream, enabling processing volumes ranging from several microliters to several liters.
- The flow configuration provides the capability to design a closed system, which can be sterilized prior to use and can be incorporated into cGMP manufacturing processes.
- The system does not require a controlled environment and can be operated in a routine laboratory, as is the case with blood cell separators. This makes possible cell processing at the point of care, i.e., at major medical centers that will be the primary sites for cell therapy.
- The MaxCyte System is designed for clinical use. All components that contact the cells undergoing processing are clinical grade. E.g., electrode materials are chosen so that electrolysis does not result in toxic metalions in the processed cell suspension. This requires manufacturing electrodes with materials such as gold.
- The process is rapid, permitting processing of relatively labile cells and reagents.
- The final results of the MaxCyte cell loading process depend on more than the electroporation, and extensive attention is given to pre-electroporation cell handling, processing buffers and post-electroporation cell management.

Table 1. Efficiency and viability of processed cells
Results with primary cells (blue shading) and cell lines (yellow shading). "Efficiency" is the percentage of observed cells displaying green fluorescence 24 to 48 hours after processing with a plasmid coding for GFP. "Viability" is the percentage of cells not displaying propidium iodide (PI) fluorescence (both assayed via flow cytometer).

Cell Type	Efficiency (eGFP+)	Viability (Propidium iodide+)
Human mesenchymal stem cells	80%	90%
Primary human myoblasts	90%	90%
Human umbilical cord endothelial cells	75%	90%
Human hematopoetic stem cells (CD34+)	50%	65%
Human dendritic cells	50%	80%
Primary human B lymphocytes	80%	80%
Primary human T lymphocytes	50%	70%
293 cells	90%	90%
Jurkat cells	80%	80%
Huh-7 cells	80%	80%

Capabilities

The MaxCyte system has been used to load a wide range of cell types using various biologically active molecules such as proteins, antibodies, plasmids, mRNA, and siRNA. A sampling of system performance using transfection efficiency as an indicator is presented in Table 1. Note that the "Efficiency" value is based on the expression of an enhanced green fluorescent protein (eGFP) plasmid, which is a function of cell loading and protein synthesis.

Some cells, such as T-lymphocytes, do not readily synthesize protein and a better measure of the power of the system is seen by measuring incorporation of a fluorescence-labeled macromolecule. With this procedure using, for instance, 500 kD FITC-dextran, 95 percent of cells are loaded. The standard configuration's processing rate is approximately 600 million cells per minute.[4] A modified assembly (Table 1) achieves a processing rate that is ten-fold higher than the standard configuration rate.

Therapeutic proteins produced by transfected cells function normally in biological systems with no observed toxicities. Following are the results of testing with erythropoietin and IL-12. The two test groups consisted of five mice each, and control mice received untreated cells.

(a) Mouse fibroblasts were transfected with a DNA plasmid encoding human erythropoietin (a cytokine that stimulates production of red blood cells). The cells were subcutaneously injected into BalbC/SCID mice (2 x 10^6 cells/mouse). The hematocrits (the percentage of red blood cells in blood) were measured weekly. The erythropoietin functioned normally *in vivo*. EPO mice hematocrit levels at 12 and 26 days were 49.9 +/- 3.2% and 48.5 +/-2.6%, respectively; control mice levels were 43.4 +/- 1.6% and 43.2 +/- 2.0%, respectively. By protocol, the mice were sacrificed at day 25.

(b) Mouse embryonic fibroblasts were transfected with a DNA plasmid encoding mouse interleukin 12 (IL-12), and subcutaneously injected into the right flank of C3H mice (2 x 10^6 cells/mouse). Matrigel (a biocompatible scaffold) mixed with a stimulator of blood vessel growth (basic fibroblast growth factor [bFGF]) was also injected into the right flank. bFGF initiates vessel growth and attracted red blood cells into the Matrigel, which was removed seven days post injection, homogenized, and analyzed for hemoglobin concentration. IL-12 inhibits blood vessel formation resulting in fewer red blood cells in the Matrigel of treated mice (Fig. 2).

Cells can be transfected with multiple plasmids simultaneously. Cells processed with separate plasmids coding for GFP and for red fluorescent protein, Ds-Red, highly expressed both proteins after the process. Green and red fluorescence was observed via flow cytometer and fluorescent microscopy. This capability was used in developing viral vectors, which is discussed in a subsequent section.

When larger volumes are processed, results remain consistent. When moderate volumes (e.g., 50 ml) or large volumes (e.g., 1 L) are processed, samples taken at intervals indicate similar values for efficiency and viability. Data are displayed in the section on large-scale processing.

Cells are transfected with a high level of efficiency. High efficiency was obtained with the Jurkat cell line, primary human lymphocytes, and B-cells from patients with chronic lymphocytic

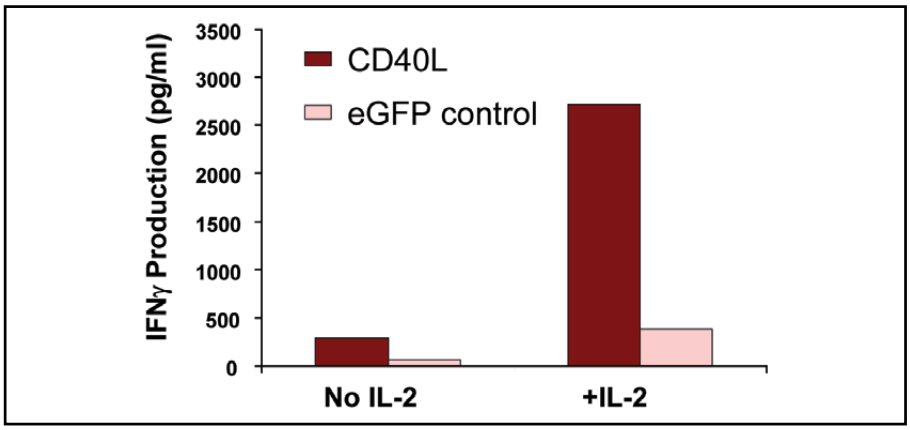

Figure 3. Expression of CD40L+IL-2 enhances stimulation of T cells. CLL cells were transfected using plasmids encoding hCD40L or GFP (control). The transformed cells were then incubated with allogeneic T cells and the production of interferon gamma used as an indicator of T cell stimulation.

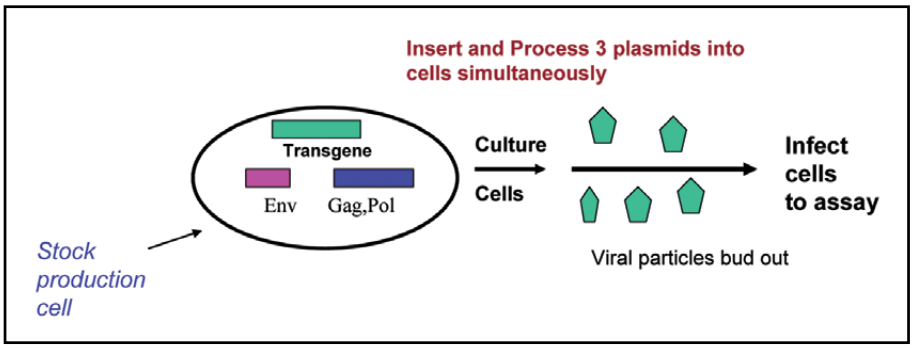

Figure 4. Production of lentiviral vectors with the MaxCyte system. "Stock production cell" refers to a standard cell line (e.g., 293 cells).

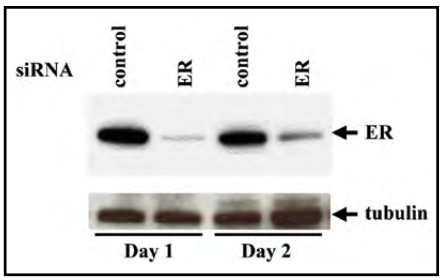

Figure 5. siRNA introduced by electroporation significantly silenced target gene expression. Human breast cancer MCF7 cells were transfected with siRNA of GFP (green fluorescence protein), a control, or with siRNA of an estrogen receptor (ER). The transfected cells were lysed at day one or two post-treatment. Target gene (ER) expression was analyzed by Western blot with anti-ER antibody. Tubulin expression revealed that an equal amount of protein was loaded in each lane. ER expression was reduced by more than 90 percent at both time points. Gene array studies showed that changes in the background gene activity were much lower than with other loading methods (e.g., cationic lipids). *(These studies were conducted at the laboratory of Dr. Paul Meltzer, National Human Genome Research Institute, National Institutes of Health [NHGRI/NIH].)*

leukemia (CLL). Data are presented in the discussion of the CLL study.

Applications

Cells that are reportedly difficult to transfect have been processed successfully using the MaxCyte system. The system has been used in the preparation of specific cell-based therapeutics, for incorporation into industrial bioprocessing, and for cell-based drug discovery and development. Examples of these applications are presented in Figure 3.

Cell-Based Gene Therapy

Electroporation enables non-viral gene delivery in an *ex vivo* mode while avoiding persistent concerns about the safety of viral vectors. Recent literature supports those concerns:

- Wu *et al.* present data which indicate that a retroviral vector selectively integrates into active gene areas, as compared to non-active areas (introns) of the chromosome. [5]

- Nakai *et al.* describe studies that indicate appreciable integration with adeno-associated viral vectors. Such integration was previously thought to occur only rarely.[6]

Clearly, alternative approaches are needed.[7] Electroporation provides an alternative that simplifies preparation and testing logistics, and treads a less onerous regulatory path.

This has specifically been demonstrated by the prompt clearance of applications through the Recombinant DNA Advisory Committee and FDA, with regard to gene delivery aspects, for the CLL trial described. In general, users of the MaxCyte system are required to provide appropriate preclinical toxicology data on the transgene employed, but not on the vector per se, in accord with the policy that derived from the *Non-Clinical Toxicology In Support of Licensure of Gene Therapies Workshop*.[8] There is a Master File with data on the MaxCyte system at CBER/FDA. This file has been referenced for IND applications.

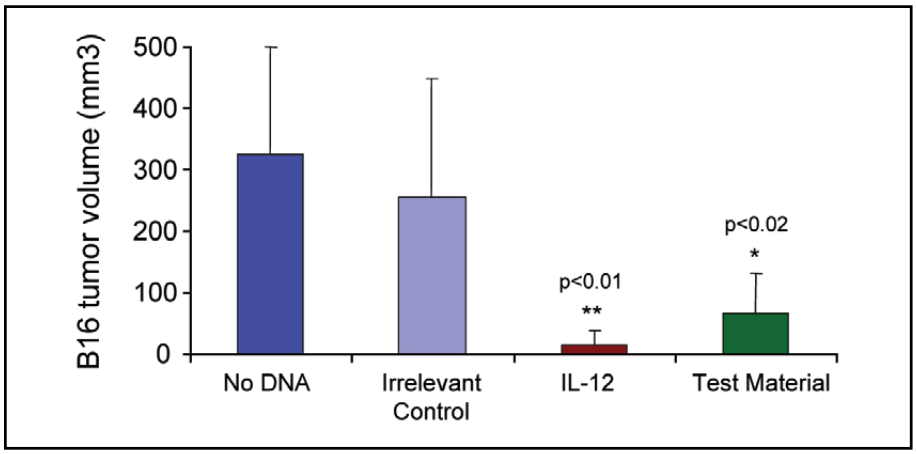

Figure 6. Screening candidate genes. B16 F10 melanoma cells were transiently transfected with various DNA plasmids. After 24 hours, mice were injected subcutaneously with 1 x 10^5 gene-modified or unmodified (no DNA) tumor cells. Tumor volumes were calculated bi-weekly for three to four weeks (day 15 is shown).

Table 2. Data generated with the very large-scale configuration.
The model system chosen for the transfection of Jurkat cells was a standard plasmid carrying full-length cDNA encoding for eGFP driven by a cytomegalovirus (CMV) promoter. Jurkat cells (2.5 x 10^{10} in a final total volume of one L) were washed and suspended in electroporation buffer and eGFP plasmid was added (100 μg/ml DNA). The flow system for this demonstration was equipped with a sample port and 1-ml samples were withdrawn at 100-ml intervals. The fluorescence-activated cell sorting (FACS) results on these samples (direct gating and counting) are summarized and compared with four static experiments.

	Very Large Scale		Static (cuvette)	
	% positive (Ave/StdErr)	% viable (Ave/StdErr)	% positive (Ave/StdErr)	% viable (Ave/StdErr)
24 hours	66 / 1.5	96 / 1.4	64 / 1.2	90 / 1.0
48 hours	78 / 1.0	97.2 / 0.8	82.6 / 0.8	91.1 / 0.8

Example 1. Immunotherapy for Chronic Lymphocytic Leukemia (CLL)

CLL, a B-cell malignancy, is the most common leukemia in adults. Leukemic cells from patients do not stimulate a significant immune response, but if CLL cells express the protein CD40L, the immune response is greatly enhanced and leads to diminished tumor mass. Clinical studies using a mouse gene to transfect autologous CLL cells with the gene for CD40L showed promising results.[9] Other studies have shown that the addition of IL-2 enhances the immune effects. The MaxCyte system delivers the genes for CD40L and IL-2 into CLL cells with very satisfactory efficiency and cell viability, and the cellular product is ready for release soon after processing.

A Phase I/II clinical study has been developed in collaboration with the Center for Cell and Gene Therapy at Baylor University. In the study, blood is obtained from patients via standard techniques, and CLL cells are separated and processed using the MaxCyte system (to a sufficient volume to yield several doses, plus a sample for testing). The blood is then frozen and stored. Six doses are administered to patients every one to two weeks, while clinical and laboratory variables are monitored for safety. Efficacy will be demonstrated by measuring the decreases in CLL cell counts and lymph node size.

Example 2. A Cell-Based Therapeutic Approach for Pulmonary Arterial Hypertension.

MaxCyte is working with Northern Therapeutics to develop a cell-based gene therapy for pulmonary arterial hypertension (PAH), a progressive and often fatal disease. Using a rat model of PAH, both rat and human dermal fibroblasts

as well as erythroid progenitor cells were loaded with a plasmid encoding endothelial nitric oxide synthase (eNOS). The transfected cells can be cryopreserved with maintenance of eNOS transgene expression upon thawing. To test *in vivo* efficacy, Fisher 344 rats, which had been treated with monocrotaline to induce PAH, received an injection through the right jugular vein of either 1 x 10^6 syngeneic fibroblasts that had been transfected with an eNOS plasmid or loaded with a null control plasmid.[10] Right ventricular systolic pressure, measured 25 days after treatment, was reduced in animals receiving eNOS cell-based gene therapy (38.5 ± 3.3 vs. 52 ± 3.9 mmHg [p=0.013, reversal]). A clinical study is in preparation.

Bioprocessing. The properties of the MaxCyte system that make it suitable for production of selected biopharmaceuticals (biocompatible materials, scalability, closed and sterile processing unit) allow incorporation of the system into cGMP manufacturing processes. The system's capability to load two or more macromolecules simultaneously has been used to produce viral particles by loading plasmids encoding the viral components into routine production cell lines.

Scaling up production of non-replicating viral vectors is a major hurdle for large gene therapy clinical trials. Transient, simultaneous transfection of cells with multiple plasmids results in the production of vectors and decreases the possibility of viral-genome recombination. Current transient transfection methods (e.g., $CaPO_4$) allow production of small volumes of viral vectors, but the process can introduce inconsistencies from lot to lot, thus raising regulatory issues. Furthermore, precipitation of $CaPO_4$ interferes with downstream purification and the concentration of viral particles. The method presented here can co-transfect large volumes of cells, thereby scaling up production to research and clinical levels. The basic elements of the process are shown schematically in Figure 4.

The system has demonstrated the capability to produce lentivirus- and alphavirus-based therapeutics on a laboratory scale. Its ability to operate on a large scale (discussed below) makes it suitable for all stages of clinical research and for commercialization. Projects with partners who are developing virus-based therapeutics are underway.

Cell-based Drug Discovery and Development. The MaxCyte system has been used in a variety of applications:

- Loading cells with immunoglobulins to block selected functions. IgG was loaded with high efficiency, and specific functions were observed to decrease.
- Rapid production of proteins from candidate genes of potential interest using transient transfection of production cell lines. This technique can yield milligram quantities of protein, allowing pilot testing before embarking on viral vector-based transfection for long-term production of promising proteins.
- Loading cells with inhibitory RNA to provide model systems for testing potential therapeutics. An example, using siRNA, is shown in Figure 5.
- *In vivo* evaluation of candidate genes by transfecting appropriate cell lines and observing the effects in animals.[11] An example of testing potential antineo plastics is displayed in Figure 6.

Large-Scale Processing

A configuration of the MaxCyte system has been developed that can process cells swiftly for very large-scale applications. Data have been generated for the transfection of one liter of cell suspension, containing 2.5 x 10^{10} cells. The configuration used to produce most of the data displayed in Table 1 can process 1 to 100 ml of cell suspension at a rate of several ml/minute, or as high as 6 x 10^8 cells/minute. The very large-scale configuration can process several liters of cell suspension in a single operation at a rate of 200 ml/minute. Summary data with this configuration are displayed in Table 2. (It should be noted that this single experiment is a demonstration of the system's volume capability and that conditions were not optimal.)

Summary

Electroporation, as used in the MaxCyte system, can effectively and reproducibly load cells with exogenous DNA or other molecules. Because the processing module is a closed, sterile unit, it can be used for point-of-care therapeutic protein production and can be incorporated into cGMP processing systems. The system's use in FDA-approved clinical studies should pave the way for its large-volume capability to be used in a wide range of bioprocessing applications.

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