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Multifunctional Cell Processing with Plasmonic Nanobubbles

Ekaterina Y. Lukianova-Hleb, Dmitri O. Lapotko

***Abstract*—**Cell processing techniques for gene and cell therapies use several separate procedures for gene transfer and cell separation or elimination, because no current technology can offer simultaneous multi-functional processing of specific cell sub-sets in heterogeneous cell systems. Using our novel on-demand nonstationary intracellular events instead of permanent materials, plasmonic nanobubbles, generated with a short laser pulse only in target cells, we achieved simultaneous multifunctional cell-specific processing with the rate up to 50 million cells per minute.

***Keywords*—**Delivery, cell separation, graft, laser, plasmonic nanobubble, cell therapy, gold nanoparticle.

I. Introduction

WHILE cell and gene therapies that have shown promise against human diseases including cancer require *ex vivo* they critically depend upon processing of human cell grafts in order to eliminate unwanted cells from a heterogeneous cell suspension and to genetically modify one or more cell subsets to increase their therapeutic efficacy. Ideally both elimination and transfection should be highly efficient, cell-specific and fast. Existing methods [1]-[18], however, lack such characteristics, especially those of multi-functionality and cellular selectivity when applied to heterogeneous cell systems. As a result, current cell processing for cell and gene therapies is often slow, expensive and labor intensive and is compromised with high cell losses and poor selectivity, thus limiting the efficacy and availability of these cell therapies.

We developed an entirely new approach that achieves the simultaneous transfection of target cells and the elimination of unwanted sub-sets of other cells in heterogeneous grafts in one procedure. This procedure is activated on-demand with a short laser pulse that selectively generates in target cells newly developed class of tunable multi-functional transient events, called plasmonic nanobubbles (PNBs) [19]-[24]. A PNB is not a particle but a transient nanosecond event, a vapor nanobubble that is generated around a gold nanoparticle (NP) after it absorbs a short laser pulse, converts its energy into heat and evaporates its liquid environment in a nano-explosive manner (Fig. 1). We recently demonstrated that PNBs allow optical detection, trans-membrane injection of molecular cargo to and the immediate destruction (elimination) of specific target cells with high speed, selectivity and without collateral damage even when the majority of cells are non-target [20]-[24]. The specific function, payload delivery or destruction, is determined by the maximal size of the PNB (Fig. 1), which, in turn, is determined by the NP's properties and by the energy of the laser pulse [19]-[24]. We hypothesized that the ability of each NP type to generate PNBs of different sizes under identical optical excitation coupled with the cell-specific targeting and clustering of NPs conjugated to cell-specific antibodies would allow the simultaneous transfer of molecular cargo into gold sphere-targeted cells and the destruction of gold shell-targeted cells in a simultaneous bulk treatment of a heterogeneous cell system with high efficacy, speed and selectivity and with low toxicity (Fig. 1). This technology would create a universal platform for cell and gene therapy including stem cell transplantation. To test this hypothesis we experimentally studied responses of different cells *in vitro* to targeting with specific NP types and to a simultaneous bulk treatment with a single laser pulse that generated PNBs in those cells.

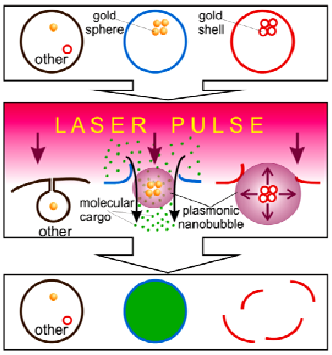

Fig. 1 Multi-functional cell-specific processing with plasmonic nanobubbles (PNBs) that are selectively generated around the clusters of gold spheres in spheres-targeted cells (*blue*) and around the clusters of gold shells in shells-targeted cells (*red*) with a single laser pulse, resulting in the simultaneous delivery of molecular cargo into blue cells due to injection of the molecules (green dots) with small PNB and mechanical destruction of red cell with large PNB without the damage to other cells, all realized in a single pulse treatment

II. Results

A. PNB Generation in Gold NP-Treated Cells

The maximal size of a PNB determines its specific cellular effect, destruction or delivery. Large PNBs mechanically destroy cells while small, non-invasive PNBs inject

E.Y. Lukianova-Hleb is with the Rice University, Houston, TX 77005 USA (e-mail: dmitri.lapotko@rice.edu).
D.O. Lapotko is with Rice University, Houston, TX, 77005 (phone: 713-348-3708; fax: 713-348-5154; e-mail: katsiaryna.hleb@rice.edu).

extracellular molecules into the cell. We initially studied the ability of cells to generate PNBs of two different sizes under exposure to a single laser pulse. We applied two NP types with different PNB generation efficacies, 60 nm hollow gold nanoshells (NS) that generate maximal PNBs and 60 nm solid gold spheres (NSP) that generate much smaller PNBs under identical optical excitation conditions [19]. Both NP types were used in plain and OKT3 antibody-conjugated forms (OKT3 antibody recognizes CD3 receptor expressed in the employed J32 model cells [20], [21], [24] and is also employed in clinic for manipulation with human T-cells). The NS-OKT3-targeted J32 cells were labeled with red fluorescent marker (Calcein Red AM) and the NSP-OKT3-targeted J32 cells were labeled with blue fluorescent marker (DAPI) for their identification through fluorescent imaging. After incubating the cells for one hour with each type of NPs, the cells were mixed (Fig. 2 (a)) and individual cells were exposed to a single laser pulse (70 ps, 532 nm, 0.22 mm) of a specific fluence above the PNB generation threshold in order to generate and detect PNBs.

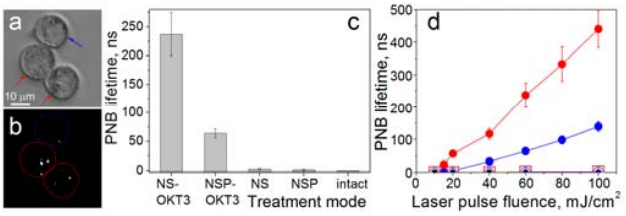

Fig. 2 PNBs in cells targeted with gold spheres (NSP) and shells (NS). (a): Bright field image of a mixture of J32 cells treated by NS-OKT3 (*red* arrow) and NSP-OKT3 (*blue* arrow); (b): optical scattering time-resolved image of large (bright) PNBs in NS-OKT3-treated cells (*red*) and small (dim) PNBs in NSP-OKT3-treated cells (*blue*); (c): PNB lifetime in NS-OKT3, NSP-OKT3, NS, NSPs -treated and intact cells, all obtained under exposure to a single laser pulse of 60 mJ/cm^2; (d): PNB lifetime as a function of the laser pulse fluence for NS-OKT3 (*solid red*), NSP-OKT3 (*solid blue*), NS (*hollow red*), NSP (*hollow blue*) -treated and intact (*solid black*) cells

Transient PNBs in individual cells were detected and imaged with time-resolved optical scattering imaging by using a pulsed probe laser. The light scattered by the PNBs produced their bright images (Fig. 2 (b)). PNB lifetimes were analyzed for five cell populations under identical optical excitation: intact cells, cells incubated with plain NSs and NSPs and cells incubated with OKT3-conjugates of NSs and NSPs (Fig. 2 (c)). In the range of laser pulse fluence between 10 mJ/cm^2 (close to the PNB generation threshold) and 100 mJ/cm^2 we observed PNBs only in cells treated with OKT3-conjugated NPs (Fig. 2 (c), (d)). Intact cells or cells incubated with plain NPs did not produce any PNBs at all because the PNB generation threshold in those cells was apparently higher than the laser fluence applied. In contrast, the cells incubated with the same NPs conjugated to the CD3-specific antibody OKT3 showed a 92-96% probability of PNB generation because their PNB generation threshold fluences were lower than the fluence applied. Such a significant reduction in the PNB generation threshold fluence (compared to cells targeted with plain NPs) was caused by the formation of large intracellular clusters of NPs through the mechanism of the receptor-mediated endocytosis [20]-[25]. We have found earlier that an increase in the NP cluster size reduces the PNB generation threshold fluence that will be the minimal for large intracellular NP clusters and maximal for single non-specifically taken NPs [20]-[24]. Thus, the receptor-mediated endocytosis of NP-OKT3 created the largest NP clusters in CD3-positive cells and enabled the selective generation of PNBs under exposure to laser pulses of low a fluence level starting at 10-15 mJ/cm^2 (Fig. 2 (d)).

B. Flow Treatment of a Heterogeneous Cell System with a Single Laser Pulse

The efficacy of the PNB flow treatment and safety of PNBs were evaluated for heterogeneous cell suspension of non-target, destruction target and injection target J32 cells after their pre-treatment with NS-OKT3 (destruction target cells) and NSP-OKT3 (injection target cells). The destruction target and injection target cells were labeled also with two fluorescent markers (DiA and DiR) to identify the cells and to monitor their viability after PNB generation. Then 3 cells populations (non-target, destruction and injection target J32 cells) were mixed into one suspension to model a heterogeneous cell system and green fluorescent dye (FITC-Dextran) was added to the cell suspension as a model of the molecular cargo. Next, the suspension was pumped through a flow cuvette and treated with laser beam for the bulk PNB treatment (Fig. 3). We used the primary data to set the pulse fluence to the level of 33 mJ/cm^2 that corresponds to the PNB lifetime of 30-70 ns in NSP-OKT3-treated cells and > 200 ns in NS-OKT3-treated cells under expose by the single laser pulse (532 nm, 20 ps) (Fig. 4 (a)). The broad laser beam simultaneously and identically irradiated hundreds of mixed cells in one pulse. All the cells received a single pulse exposure. After the laser treatment, FITC-Dextran was immediately washed off and a flow cytometry evaluation of laser- and NP-treated cells was done.

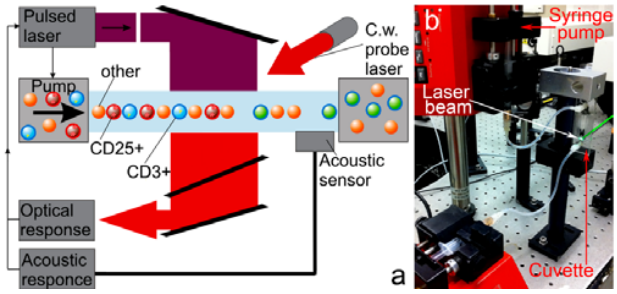

Fig. 3 (a) Functional diagram of the PNB flow system with the pulsed broad excitation laser (*purple*), sterile flow cuvette with the pump and reservoir, and two PNB monitoring paths: optical time-response is detected with continuous (*red*) laser and acoustic response is detected with acoustic sensor; (b): Photo of the designed PNB flow system with disposable flow cuvette connected to dispersing and collecting pumps and exposed to the pulsed laser

We have found that small PNBs of 30-70 ns provide efficient non-invasive transfer of external molecular cargo (Fig. 4a, b). Larger PNBs of > 200 ns lifetime efficiently destroy the cells (Fig. 4 (a), (c)). The developed experimental system delivered >90% injection efficacy, >99% cell destruction efficacy with 99% specificity and 96% viability of injected and non-target cells (Fig. 4, Table I).

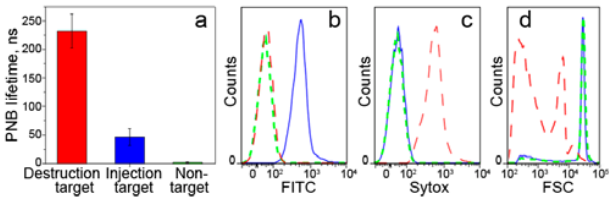

Fig. 4 Single laser pulse (532nm, 20 ps, 33 mJ/cm^2) treatment of three cell types. (a): Population-averaged PNB lifetime obtained from individual cells for three cell populations: non-target (*green*), destruction target (*red*, treated with NS-OKT3) and injection target (*blue*, treated with NSP-OKT3). Flow cytometry evaluation of laser- and NP-treated cells show the histograms for destruction target cells (*red*), injection target cells (*blue*) and non-target cells (*green*): (b): Injection efficacy as FITC fluorescence intensity; (c): Cell viability as Sytox fluorescence intensity; (d): Cell viability as forward scattering intensity

TABLE 1
POPULATION-AVERAGED PARAMETERS OF THREE CELL GROUPS AFTER THE PNB FLOW PROCESSING AT THE RATE OF 45 MLN CELL/MIN

Cell type	Injected cells, %	Survived cells, %	PNB lifetime, ns
Destruction target	-	0.5±0.3	232±30
Injection target	90±5	96±4	46±15
Non-target	0	100	1.9±1.1

This experiment demonstrates a simultaneous activation of two different cellular functions of PNBs, the non-invasive delivery of molecular cargo and the destruction of cells. In addition, we observed a single cell selectivity of both functions in a heterogeneous cell suspension. Finally, these two functions were rapidly administered in a single laser pulse bulk treatment of multiple cells in less than a nanosecond. Such high efficacy, selectivity and speed were provided by the high cellular specificity of PNBs and their on-demand mechanical (not thermal) mechanisms of the cell destruction and cargo delivery. Such multi-functional and selective processing of a heterogeneous cell system in a single bulk treatment was never achieved beforehand with existing cell processing methods.

III. Discussion

A. Comparison of the PNB Mechanisms with Current Approaches

Current *ex vivo* methods of cell processing for gene and cell therapies use several separate procedures for gene transfer and cell elimination/separation and enrichment, because no current technology can offer simultaneous, multi-functional processing of different specific target cells in highly heterogeneous cell systems. Our first major innovation is to use a single process, PNB generation, with multiple functions to simultaneously engineer one target cell subset, eliminate a second, and leave the non-specific bulk cells unmodified. The second innovation is in providing single cell specificity and a high speed of processing of a heterogeneous cell system.

Cell destruction (elimination, separation) methods use physical and affinity criteria such as filtering, centrifuging, fluorescent-activated flow sorting, and magnetic and adsorbent removal of target cells. The best results were achieved with target-specific antibodies conjugated to either magnetic beads or biotin to bind to the target cells and then to pass through columns to select the target cells (for example CD34+ stem cells [1]-[3]). When applied to human grafts, the limitations of immunotargeting are in the incomplete removal of contaminating cancer cells or the removal of important immune cells [1]-[3], as well as the lack of selectivity due to unavoidable non-specific binding of antibodies to non-target cells [1]-[3]. None of these technologies can provide the optimal combination of efficacy, selectivity, processing rate and low toxicity for processing heterogeneous cell systems required in clinic.

Cell injection and transfection methods are similarly limited. Three major approaches deliver plasmids with viral, non-viral using plasmid carriers and non-viral using external energy methods [4-18]. While viruses offer greater efficacy of gene transfer, non-viral methods provide better safety and are usually less immunogenic. Carrier-based approaches use liposomes, dendrimers, polyplexes, polyethyleneimine and other nanoparticles. Of these methods, lipofection (liposomes as carriers) is widespread [7], [8]. Use of plasmid carriers improves the efficacy and safety of gene transfer [7]-[11], but the selectivity of such methods in heterogeneous cell systems is limited by the non-specific uptake of carriers by non-target cells. PNBs with their much higher cellular specificity overcome this limitation because they do not depend upon the non-specific uptake of PNB-generating gold nanoparticles [20]-[24].

External energy-based methods use sono-, electro- and

opto-poration of cells, of which electroporation/nucleofection is most widely used, despite its poor selectivity and cellular toxicity [12]-[14]. Gene transfer also employs laser methods through heating, shockwave generation, optical breakdown and bubble generation [15]-[18]. The bubbles originate from external thermal or cavitation sources and thus they cannot discriminate target from non-target cells. Also, no current laser methods provide target cell specificty in heterogenous cell systems. Moreover, almost all external energy-based methods depend on the slow diffusion of plasmids through the entry point produced in the cell membrane. Rapid active delivery through opto-injection can be achieved for individual cells by using femtosecond laser pulse-induced optical breakdown [16] but requires the precise positioning of the laser beam on an individual target cell and therefore cannot be used for bulk treatment with high processing rates.

In contrast to the above limitations, we demonstrated several innovative properties and advantages of PNBs that were validated in our preliminary studies: (1) High cellular specificity (10-20 fold better than that of gold NPs) due to the cluster-threshold PNB mechanism that prevents the generation of PNBs in non-target cells under the simultaneous optical excitation of many target and non-target cells [20]-[24]; (2) Active nanosecond mechanical, non-thermal, and reagent-free mechanisms of the delivery of molecular cargo or cell destruction with the highest precision of cell treatment [20]-[23]; (3) Low biodamage to non-target cells due to the localized nature and high target cell specificity of PNBs [21]-[23], high cell-processing rate, > 10^8 cell/min (especially compared to fluorescent-activated cell sorters, top of the line equipment among current technologies). All the above performance is achieved under low doses of gold nanoparticles and laser energy, three to six orders of magnitude less than in current gold nanoparticle-based methods of gene delivery and photothermal destruction of cells. Based on these innovative features of PNBs, we will develop several entirely novel elements: (1) Multi-functionality is based on the cell subset-specific generation of PNBs of two different sizes under simultaneous treatment of a heterogeneous cell system with a single laser pulse. This will provide gene transfection of T- and destruction of unwanted Treg- cells with real-time guidance. Such three-in-one functionality is not supported by any other technologies used alone. (2) High speed and single target cell specificity of graft processing will use the combination of rapid intracellular mechanisms with bulk flow treatment with a broad laser beam that simultaneously irradiates thousands of cells while generating PNBs only in specific target cells.

B. Applications

The multi-functionality, selectivity and speed of the described PNB method will find several clinical applications for cell and gene therapy and stem cell or bone marrow transplantation. The ability to simultaneously genetically modify target cells (like T-cells) and eliminate unwanted cells (like regulatory T-cells) from a highly heterogeneous graft, with single cell selectivity and without compromising other important accessory cells, will enhance the effectiveness of cell engineering in general and gene therapies in particular. The technology may subsequently be applied to process any liquid tissues to improve the outcome of other cell-based interventions in cancer and other disorders. The high speed of the PNB mechanisms coupled with the broad laser beam (available with commercial lasers) will enable a cell processing rate of up to 10^9 cells per minute, which will meet clinical requirements for cell processing.

IV. Conclusions

Using the cell-specific generation of plasmonic nanobubbles of different sizes we achieved a simultaneous multifunctional cell-specific processing in a rapid single pulse procedure in a heterogeneous cell suspension: delivery of external molecular cargo to one type of cells and the concomitant destruction of another type of cells. None of the existing methods of cell processing can provide such simultaneous and cell-specific multi-functionality. This multi-functionality was realized through generating cell-specific transient plasmonic nanobubbles of two cell type-specific sizes around intracellular clusters of two types of gold NP conjugates with different PNB generation efficacy. Single laser pulse bulk treatment of multiple heterogeneous cells showed high efficacy and selectivity in both the delivery of molecular cargo and cell destruction without damaging non-target cells. The optical detection of PNBs enabled two additional functions of the detection of specific cells and of a real-time guidance of their processing. Thus the developed PNB method supports four functions in a single laser pulse procedure: cell detection, delivery of molecular cargo, cell destruction and the guidance of delivery and destruction.

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