Gold-coated Liposomes Encapsulating Signaling Molecules for Initiating Cellular Communication

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ABSTRACT

Cell signaling in the tumor microenvironment is critical for the initiation, progression and metastasis of cancer. To better understand these communication pathways and therefore better design and develop therapies and diagnostics for cancers, experimental methods of probing cellular communication at a single to multi-cell level are needed. Here, we manipulate gold-coated liposomes encapsulating signaling molecules using an optical trap to initate cell signling cascades. The high polarizability of the liposome's unique gold shell allows stable optical trapping for subcellular manipulation in the presence of cells. Specifically, we encapsulated the cell signaling molecule inositol trisphosphate (IP3), a ubiquitous intracellular secondary messenger involved in GPCR and Akt signaling cascades, within 100 nm gold-coated liposomes and used an optical trapping laser to deliver liposomes into the cytosol of a single cell to initiate localized cell signaling. Upon optical injection of liposomal IP3 into a single ovarian carcinoma cell, we observed localized activation as reported by changes in Indo-1 fluorescence intensity. With established gap junctions between the injected cell and neighboring cells, we monitored propagation of this signaling to and through nearby cells. Subsequently, we investigated the ability to modulate cell signaling by, for example, varying the number of IP3-containing gold-coated liposomes injected into a single cell. By combining optical trapping with gold-coated liposomes encapsulating signaling molecules, we present a unique *in vitro* tool for studying cell signaling within the tumor microenvironment.

Keywords: liposomes, gold nanoparticles, cell signaling, cancer, optical trap

1 INTRODUCTION

Cancer is the second leading cause of death in the United States and third in the world [1]. Major improvements to how this group of diseases is diagnosed and treated are hindered by our poor understanding of the intricacies of the tumor microenvironment. Specifically, cellular communication, involving both cancer cells and the host's own healthy cells, is critical for the initiation, progression and metastasis in cancer. Dysfunctional cell communication combined with co-opted normal cell signaling pathways make treating this group of diseases especially difficult, a major contributor to drug resistances, malignancies, and high recurrence rates in many cancer types [2]. To better understand these communication pathways, and therefore better design and develop therapies and diagnostics for cancers, we develop research tools for studying cell communication at a single cell level. Here, we present a novel benchtop research tool that combines multifunctional composite nanocapsules with optical trapping to interrogate cell signaling propagation among individual cells. We encapsulate endogenous signaling molecules in 100 nm gold-coated liposomes engineering to interact with a highly focused infrared trapping laser, allowing for manipulation and delivery of the nanocapsules at a cellular to sub cellular level. Such tools enable studying signaling events at unprecedented spatial and temporal resolution.

1.1 Tumor Microenvironment

Recently, cell communication in the tumor microenvironment has been recognized as a more significant player than previously thought [2]. One commonnly observed characteristic of cancer cells is their loss of communication via gap junctions [3], which normally act as cell signaling conduits connecting nearby cells, thus indicating a possible target for improving cancer therapeutics [4]. As an example of communication pathways in cancer, and to demonstrate the utility of our optical injection system, we examine the role of a gap junction-mediated signaling cascaded initiated by inositol trissphosphate (IP3), a ubiquitous secondary messenger in G-coupled protein receptor (GPCR) and receptor tyrosine kinase (Rtk) cell signaling pathways. IP3 targets the InsP3 receptor and calcium channel housed on the endoplasmic reticulum, which, when activated by free cytosolic IP3, amplifies the signal by rapidly dumping calcium into the cytosol. This increase in cytosolic calcium concentration, which itself acts as a secondary signaling messenger, can spill over into neighboring cells joined by gap junctions and induce calcium release in the adjoining cells. Changes in cytosolic calcium concentrations can be monitored by calcium-sensitive fluorescent dyes via fluorescent microscopy and can be used as a real-time reporter of cell signaling events. The study here used Indo-1 AM to report intracellular calcium concentration fluctuations and

therefore monitor cell signaling activity. This fluorescent dye is cell-membrane permeable until cleaved in the cell's cytosol, confining the dye molecules intracellularly and providing a reporter for calcium concentration fluctuations localized within single cells. Indo-1 has a peak excitation at 338 nm and has two distinct emission peaks, at 400 nm and 475 nm, that decrease or increase, respectively, with increasing calcium concentration.

1.2 Gold-coated Liposomes

As we previously introduced, gold-coated liposomes offer unique properties enabling encapsulation and controlled delivery of soluble agents such as drugs and contrast agents (Figure 1a), providing a platform technology for therapeutic and diagnostic delivery systems [5]. Additionally, these composite nanocapsules can be made to selectively interact with light in the visible to near infrared range (Figure 1b) providing a mechanism for controlled content release [6] which can be tuned to achieve wavelength-selective release of multiple compounds [7].

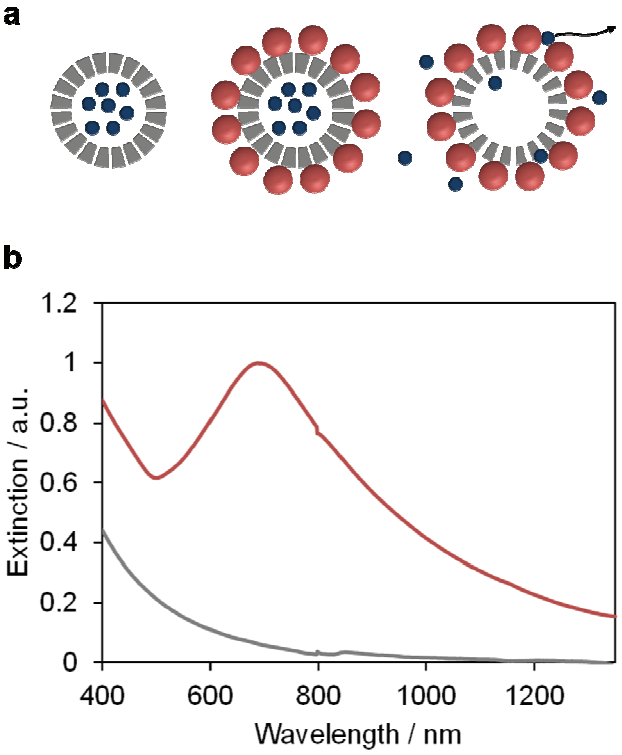

Figure 1: Gold-coated liposomes. (a) 100 nm liposomes encapsulate a soluble molecule (left). Coating with gold nanoclusters produces a plasmon-resonant pseudo-shell (middle) which interacts with light to trigger content dumping (right). (b) Extinction spectra of uncoated liposomes (grey) and gold-coated liposomes tuned to yield plasmon resonance at 760 nm (blue).

Recently, we have investigated the potential of these composite nanoparticles for controlled delivery of molecular agents to, or into, cells [8]. Because of the strong polarizability of the gold shell surrounding the liposome's lipid membrane, these composite nanocapsules can be trapped with optical tweezers, moved to a position on or near a cell, and triggered to dump its contents upon modulating the frequency of the pulsed trapping laser [8]. Additionally, the trapping laser can be adjusted to propel gold-coated liposomes into a single cell to deliver encapsulated molecular contents intracellularly, as demonstrate previously with the cell-impermeant fluorescent nuclear dye TO-PRO-3 [8]. We advance this paradigm by optically injecting the signaling molecule IP3 to demonstrate this optical manipulation technique's utility for interrogation cell communication pathways.

Here, we investigate signaling activity between human ovarian carcinoma cells (OVCAR-3). To probe cell-cell communication, we bypass extracellular receptors and directly stimulate the downstream intracellular receptor and calcium channel InsP3, housed on the endoplasmic reticulum where intracellular calcium is stored. We encapsulate cell-impermeant IP3, a ubiquitous secondary messenger and agonist for the InsP3 receptor, inside gold-coated liposomes and use an optical trapping laser to optically inject the nanocapsules into individual OVCAR-3 cells. If the triggered cell has established gap junctions with neighboring cells, signaling in the form of calcium wave can propagate within a network of cells and can be monitored via fluorescent microscopy.

2 MATERIALS AND METHODS

2.1 Preparation of Gold-coated Liposomes

Thermosensitive liposomes were formulated from synthetic lipids similar to a composition previously demonstrated for temperature-sensitive content release [9], consisting of dipalmitoylphosphatidylcholine (DPPC), monopalmitoyl-phosphatidylcholine (MPPC), and dipalmitoylphosphatidyl- ethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000) in a 90:10:4 molar ratio (Avanti Polar Lipids; Alabaster, AL). Lipids were dissolved in chloroform, convection dried under nitrogen gas, and remaining solvent was removed under vacuum. Dried lipids were introduced to IP3 at 500 μM concentration in phosphate buffered saline (PBS) to reach a final lipid concentration of 60 mM. Liposomes were prepared by freeze-thaw cycling and extrusion through 100 nm porous polycarbonate membranes. Free IP3 in the solution outside of the newly formed liposomes was removed via dialysis using 100,000 MW cut-off cellulose membranes (Spectrum Laboratories; Rancho Dominguez, CA). Resulting liposome size distributions were measured with a Malvern Zetasizer Nano ZS.

To form the plasmon resonant gold pseudo-shell on the surface of the IP3-loaded liposomes, 100 mM gold chloride followed by 500 mM ascorbic acid was added to the liposomal suspensions. This process is similar to the

technique previously reported by Troutman et al. in 2008 [5]. The presence of the desired plasmon resonant structure was immediate visualized by a change in solution color. Peak plasmon resonance was confirmed via absorption spectra obtained on a Cary 5 spectrophotometer.

2.2 Cell Culture

Cells used for these experiments were OVCAR-3 (ATCC, Manassas, Virginia). OVCAR-3 cells were subcultured under standard cell culture conditions in RPMI medium supplemented with 20% fetal bovine serum. For optical injection experimentation, confluent cells were trypsinized and seeded onto 18 mm round glass coverslips at a 1:5 split ratio and grown for 5 days in standard culture conditions prior to experimentation. OVCAR-3 cells used here were passage number 25.

2.3 Optical Trap Setup

The optical trap setup brings a trapping laser into an inverted microscope (IX71, Olympus, Center Valley, Pennsylvania) and through a 60X 1.42 NA oil immersion lens to create the highly focused trapping beam. The laser is a continuous wave 1064 nm TEM_{00} (Ventus IR, Laser Quantum, United Kingdom) with a 1.2 W maximum power, 1.2 M^2 value, and high beam pointing and power stability. To modulate the trapping beam, we use a Pockels cell (360-80 LTA, Conoptics, Danbury, Connecticut) controlled by a pulse generator (9530 Series Pulse Generator, Quantum Composers, Bozeman, Montana) driven by a voltage amplifier (25D Driver, Conoptics, Danbury, Connecticut). This light modulation system is capable of producing pulses as short as about 20 ns and frequencies up to 20 MHz allowing for efficient trapping with little heat accumulation at the thermosensitive liposome. The position of the optical trap is controlled by two beam steering lenses (f = 250 mm, Thorlabs, Newton, New Jersey) keeping one lens stationary and moving the other lens along the X, Y, and Z axes with three motorized actuators. Samples were imaged onto an air-cooled 512x512 pixel back-thinned EM-CCD digital camera (Hamamatsu, Bridgewater, New Jersey) with a protective 1064 nm OD 6 notch filter (Edmund Optics, Barrington, New Jersey) before the detector.

3 RESULTS

The results indicate that optical injection of gold-coated liposomes containing IP3 induces a cell signaling event at a single cell. By optically injecting gold-coated liposomes into a cell, we show that we can initiate a cell signaling event that spreads to nearby cells.

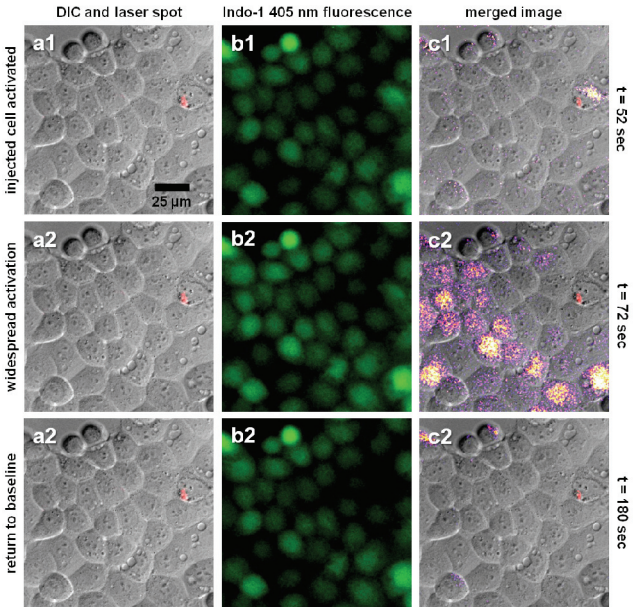

Figure 2: Optical injection of IP3 and calcium signaling. To initiate calcium signaling within and between OVCAR-3 cells, gold-coated liposomes containing IP3 were introduced the extracellular buffer with a 500 ns, 2 MHz pulsed 1064 nm trapping laser focused within the cytosol of a selected cell. The column 'a' shows DIC images of the experimental field of view with the optical trapping laser position overlaid. The column 'b' shows the 405 nm Indo-1 fluorescence images (false color) and column 'c' shows the a merged images of the calculated calcium signal with the DIC image and laser spot. Row '1' at 52 seconds into injection, row '2' is 20 seconds after the first cell is activated, and row '3' shows a return to baseline calcium signal. Scale bar = 25 µm, applies to all images.

The timing of the initiated calcium wave can be seen in the fluorescence and processed images in Figure 2. The cells selected for analysis and their locations in the field of view are shown in Figure 3a. When plotted (Figure 3b), the intensities of the analyzed cells reveal the sequential and rapid spreading of the calcium signal that appears to amplify as it progresses.

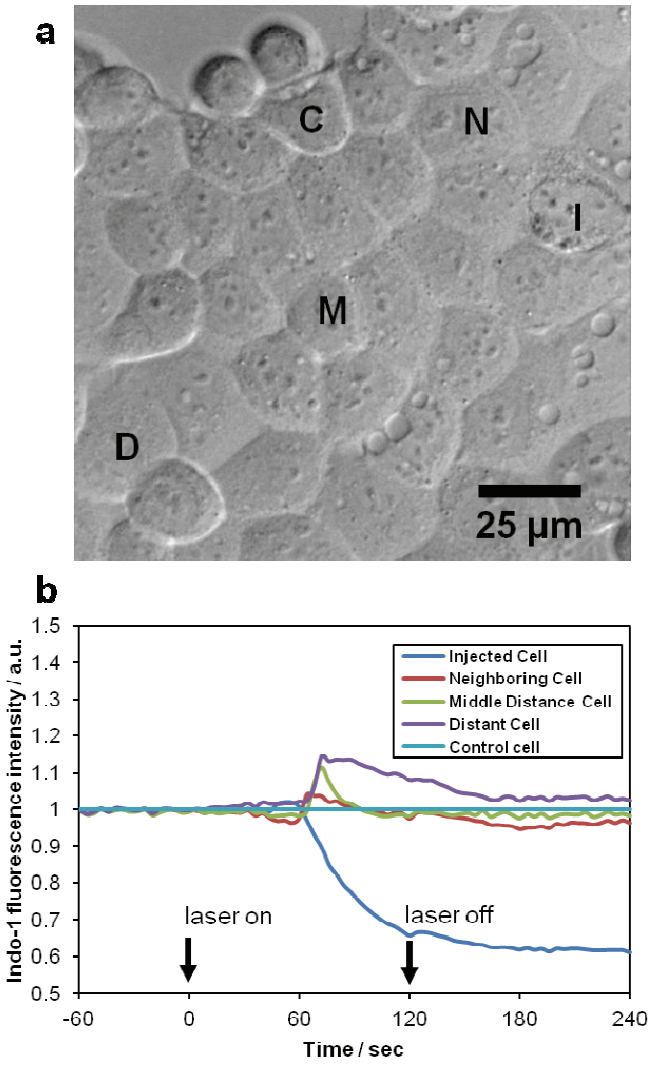

Figure 3. Progression of calcium signaling in OVCAR-3 cells. (a) The above image shows OVCAR-3 cells imaged in DIC immediately prior to optical injection. Calcium signaling was monitored over time in 5 cells labeled 'I' for the injected cell, 'N' for an adjoining neighboring cell, 'M' for a cell at a middle distance away, 'D' for a distant cell, and 'C' for the control cell. Bar = 25 µm. (b) The plot shows the timing of the measured calcium signaling of the five OVCAR-3 cells before, during, and after optical injection. The cells were continuously illuminated with 340 nm light and the 405 nm peak Indo-1 emission was continuously imaged with the EMCCD. After 60 seconds, the optical injecting laser was turned on for 120 seconds and then off for the remaining 120 seconds. All intensities normalized to the average intensity of a non-responsive control cell within the field of view during the same.

4 CONCLUSIONS AND DISCUSSION

In summary, we have demonstrated the use of a novel research tool for studying cell communication. The ability to selectively initiate a signaling event in a single cell in a small population and subsequently monitor communication to and through local cells provides a novel tool for interrogation cell communication. In the example presented here, this tool specifically shows utility for studying communication through gap junctions.

Optical injection of gold-coated liposomes encapsulating signaling molecules is a viable technique for probing cell communication, specifically in the tumor microenvironment. A better understanding of the complex signaling networks in cancer could lead to improved therapies and diagnostics.

5 ACKNOWLEDGEMENTS

This research was supported by NIH and NSF (grants CA120350, CA134424, and CBET 0853921). Additional support was provided by NIH training grants (HL007955 and EB000809).

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