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Short Communication

Real-time label-free monitoring of the cellular response to osmotic stress using conventional and long-range surface plasmons

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ABSTRACT

Cell volume and its regulation are key factors for cellular integrity and also serve as indicators of various cell pathologies. SPR sensors represent an efficient tool for real-time and label-free observations of changes in cell volume and shape. Here, we extend this concept by employing the use of long-range surface plasmons (LRSP). Due to the enhanced penetration depth of LRSP (~1 μm, compared to ~0.4 μm of a conventional surface plasmon), the observation of refractive index changes occurring deeper inside the cells is possible. In this work, the responses of a confluent normal rat kidney (NRK) epithelial cell layer to osmotic stress are studied by both conventional and long-range surface plasmons. Experiments are conducted in parallel using cell layers grown and stimulated under the same conditions to enable direct comparison of the results and discrimination of the osmotic stress-induced effects in different parts of the cell.

1. Introduction

Due to the high water permeability of the mammalian plasma membrane, an imbalance between the osmolarity of the cytoplasm and the surrounding medium triggers the redistribution of water and results in changes to the cell volume. Since disturbances in cell size and water content can result in a loss of function on the cellular and, later on, tissue and organ level, most cell types react immediately by activating diverse mechanisms for controlling and restoring the original cell volume, such as cytoskeleton restructuring or ion channel activation (Burg et al., 2007).

In the last 15 years there has been an increased demand for efficient tools regarding cell volume measurement, as numerous studies have shown the immense role of cell volume in the regulation of physiology and pathophysiology in mammalian organisms (Lambert et al., 2008). The most commonly used experimental approaches include: (1) electroanalytical methods—Coulter counting for suspended cells (Grinstein and Furuya, 1984) and impedance based volume cytometry in a microchannel for adherent cells (Ateya et al., 2005), and (2) confocal microscopy used either in combination with intra- and/or extracellular fluorophores (Allansson et al., 1999; Crowe et al., 1995; Satoh et al., 1996) or in reflection mode (Pohl and Valeyev, 2003). Although beneficial, the use of these approaches for real-time observation of living eukaryotic cells suffers from several drawbacks, such as interference of changes in the bulk conductivity with electrochemical readouts, limited sensitivity of microscopic approaches, or photobleaching and phototoxicity effects caused by the use of labels in fluorescence microscopy.

Recently, a label-free whole cell biosensor for the measurement of cell volume changes based on the phenomenon of surface plasmon resonance (SPR) was introduced (Baumgarten and Robelek, 2011; Robelek and Wegener, 2010). The penetration depth of the evanescent field of the conventional surface plasmons (cSP) is 100–400 nm, depending on the resonant wavelength (Homola, 2006), which is much smaller than the cell height. It is possible to extend the penetration depth up to several micrometers by employing more complex surface plasmon waves, such as long-range surface plasmons (LRSPs), thus providing the ability to read refractive index (RI) changes occurring farther from the SPR sensor surface (Vala et al., 2009).

In this work, we utilized both cSPs and LRSPs to study the responses of normal rat kidney (NRK) epithelial cells when they were exposed to osmotic stress. The cultured cells were transferred to the surfaces of both cSP- and LRSP-based sensors, after which cell volume measurements were performed in parallel to allow for direct comparison.

2. Materials and methods

2.1. Preparation of SPR chips

To study cell layers by cSP and LRSP modes, special plasmonic structures were designed and fabricated. A description of the SPR structures and a calculation of the penetration depths of cSPs and LRSPs are provided in the supplementary material.

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The optimum LRSP structure for investigation of NRK cells consists of an N-BK7 glass substrate, a 950 nm thick layer of Cytop fluoropolymer, 1.5 nm layer of titanium and a 30 nm gold film. For the investigation of cells using cSPs, the structure comprised of N-BK7 glass substrates, a 1.5 nm layer of titanium and a 50 nm layer of gold. The procedures used for fabrication of these SPR structures have been previously described (Vala et al., 2009). Note that for the LRSP chips used in this study, Cytop (CTL 809M, Asahi glass company, Japan) was used instead of Teflon AF to better match the refractive index of the cells.

2.2. SPR sensor instrumentation

SPR measurements were performed simultaneously on two four-channel SPR devices developed at the Institute of Photonics and Electronics, Prague. The instruments are based on spectroscopy of surface plasmons in the Kretschmann geometry of the attenuated total reflection and have been previously described (Vala et al., 2009).

2.3. Solutions

All buffers were based on Dulbecco's phosphate buffered saline solution including 0.5 mM $MgCl_2 \cdot 6H_2O$ and 1 mM $CaCl_2 \cdot 2H_2O$ ($DPBS^{++}$, Sigma-Aldrich, Germany). For hypertonic stimulation the osmolarity of the $DPBS^{++}$ buffer was increased by addition of sucrose (Serva, Germany). To obtain hypotonic conditions, a decrease in osmolarity was obtained by diluting the $DPBS^{++}$ buffer with ultrapure water (MembraPure, Germany). Osmolarities of all solutions were determined using an Osmomat 030 Osmometer (Gonotec, Germany).

2.4. Cell culture

The epithelial cell line NRK-52E cloned from a mixture of normal rat kidney cells was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). Propagation, culture and passage of the cells was performed as previously described (Baumgarten and Robelek, 2011).

2.5. Cell culture on SPR sensors

Prior to cell seeding, both SPR sensor surfaces (supporting cSPS or LRSPs) were rinsed with 70% ethanol for 10 min and dried with a stream of pure nitrogen. Subsequently the SPR chips were placed in standard 50×21 mm^2 culture dishes (TPP, Switzerland) and sterilized for 45 s in Argon plasma (Diener electronic, Germany). Immediately after removal from the plasma, the SPR chips were covered with 2 ml of DMEM culture medium containing 20 mM HEPES (Sigma-Aldrich, Germany).

NRK cells from stock culture were removed from a 25 cm^2 culture flask by standard trypsination and suspended in 4 ml of complete DMEM culture medium containing 20 mM HEPES for pH maintenance under CO_2 independent conditions. The cell suspension was added dropwise onto the sensor surface. The sensor/cell system was incubated for at least 72 h at a temperature of 37 °C. Cell growth and confluency was checked by standard phase contrast microscopy.

2.6. SPR experiment

Cell-coated SPR chips were removed from the DMEM medium and immediately mounted to the SPR system, after which isotonic $DPBS^{++}$ buffer was then injected into the flow-cell to prevent the cells from drying. After achieving a stable baseline (~30 min), cells were stimulated by changing the osmolarity of the $DPBS^{++}$ running buffer. Hypotonic stimulation was performed using a set of $DPBS^{++}$ buffers diluted with ultrapure water to 87.5% (256 mOsm/kg; $\Delta = -39$ mOsm/kg) and 75% (218 mOsm/kg; $\Delta = -77$ mOsm/kg) of its original concentration. For hypertonic stimulation, NRK cells were incubated in a $DPBS^{++}$ buffer containing additional 25 mM (318 mOsm/kg; $\Delta = +23$ mOsm/kg) or 50 mM sucrose (342 mOsm/kg; $\Delta = +47$ mOsm/kg). Each stimulation step took 20 min, after which the isotonic $DPBS^{++}$ buffer was pumped through the flow-cell again until the equilibrium was reached. Typically, four consecutive stimulations were performed on one sensor chip before the non-isotonic stimulations showed any indications of irreversibility. To check the reproducibility and reversibility of the osmotic challenges, two consecutive osmotic stimuli of equal strength were applied in the same channel. The relative error of the resulting responses was lower than ±10% (data not shown). In order to compensate for the drift of the sensor response (typically less than 10 nm/h), which is not associated with the response of the cells to osmotic stress, a reference channel was used and subtracted from the measurement channel in all experiments. In the reference channel isotonic $DPBS^{++}$ buffer was flowed across the cells throughout the whole experiment (instead of injecting the non-isotonic buffer). All experiments were performed at a temperature of 37 °C and a flow rate of 20 µl/min.

3. Results and discussion

3.1. Monitoring cell volume change using conventional and long-range surface plasmons

The time profiles of the resonance wavelength ($\Delta\lambda_{SPR}$) obtained in our experiments using cSP and LRSP structures (Fig. 1) show similar trends during non-isotonic stimulations. After starting the hypertonic stimulation, the resonant wavelength increases rapidly until a new equilibrium is reached, typically within 3–4 min. Exchange of the stimulation buffer for the isotonic $DPBS^{++}$ running buffer brings the sensor response back to the baseline level, reaching an equilibrium state in approximately the same time. The most striking difference in the sensor response obtained using a cSP-based (Fig. 1a) and LRSP-based (Fig. 1b) sensor can be observed immediately after changing buffer osmolarity. The response of the cSP-based sensor shows an immediate transient response ("spike") that precedes the main step-like sensor response and points in the opposite direction with respect to the eventual equilibrium state. These transient spikes are not present when hypertonic stimulation is monitored by LRSP (Fig. 1b). Due to the different bulk sensitivities of cSP and LRSP modes, the equilibrium responses of LRSP-based sensors are approximately three times larger than those obtained using the cSP-based sensors.

Monitoring of hypotonic stimulations of NRK cells attached to cSP- and LRSP-based sensors was performed by incubating the cells with diluted $DPBS^{++}$ buffers. These stimuli provide the inverse sensor response compared to the hypertonic stimulation. In addition, responses of both the cSPs and LRSPs exhibit transient spikes followed by a step-like change of the signal leading to a new equilibrium value (Fig. 2). Similar to the hypertonic stimulation case, a reset of the buffer conditions to isotonic osmolarity causes the signals to return to the original baseline level.

When comparing the transient spikes at the beginning and the end of non-isotonic stimulations, it can be seen that there are considerable differences between cSP- and LRSP-based sensorgrams. In the experiments utilizing cSPs, we observe pronounced spikes at the beginning and end of both hypertonic and hypotonic stimulations. Spikes are more pronounced when the osmolarity of