Plasmonic Nanobubbles: Tunable and Transient Probes for Cancer Diagnosis, Therapy and Theranostics

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ABSTRACT

Combining diagnostic and therapeutic processes into one (theranostics) and improving their selectivity to cellular level may offer significant benefits in various research and disease systems and currently is not supported with efficient methods and agents. We have developed a novel method based on the gold nanoparticle-generated transient photothermal vapor nanobubbles, that we refer to as plasmonic nanobubbles (PNB). After delivery and clusterization of the gold nanoparticles (NP) to the target cells the intracellular PNBs were optically generated and controlled through the laser fluence. The PNB action was tuned in individual living cells from non-invasive high-sensitive imaging at lower fluence to disruption of the cellular membrane at higher fluence. We have achieved non-invasive 50-fold amplification of optical scattering amplitude with the PNBs (relative to that of NPs), selective mechanical and fast damage to specific cells with bigger PNBs, and optical guidance of the damage through the damage-specific signals of the bubbles.

Keywords: nanoparticles, laser, plasmonic nanobubbles, theranostics.

1 INTRODUCTION

Combining diagnosis and therapy in one process is an emerging biomedical method referred to as theranostics [1,2]. A distinct goal of theranostics is to selectively target specific (diseased) tissues or cells to increase diagnostic and therapeutic accuracy. The major promise of the theranostics is to bring together key stages of a medical treatment such as the diagnosis and therapy, and thus to make a treatment shorter, safer and more efficient. However, this goal requires adequate tools with a high multi-functionality and selectivity.

Several theranostic methods have employed nanoparticles (NPs) as the carriers of diagnostic agents and drugs [3-5]. However, NPs themselves may also act as the multifunctional agents due to their unique properties, such as the plasmon resonances of noble metal NPs and without chemical loads. Plasmon resonances can be activated optically and convert incident light into the scattered (optical) and absorbed (thermal) components with potential for diagnostic and therapeutic applications.

We hypothesized that a combination of the photothermal properties of plasmonic nanoparticles with those of the transient vapor bubbles may be a key solution of the above problems through the development of a tunable nanoscale theranostic probe that is not a nanoparticle but a nanoparticle-generated event – the plasmonic nanobubble (PNB), which combines high optical brightness with localized mechanical impact. In the current work we have studied the optical generation and detection of PNBs around gold nanoparticles in individual living cells with the focus on tuning the PNB properties in one cell and evaluating the multi-functionality of the PNB.

2 MATERIALS AND METHODS

2.1 Principle of PNB theranostics

Cell theranostics employs a tunable and transient probe vapor bubble (Figure 1) that is generated with a short laser pulse around plasmon resonant gold nanoparticles (NP), which we refer to as plasmonic nanobubbles. The PNB is a system that results from the interaction of optical radiation with a NP and its environment.

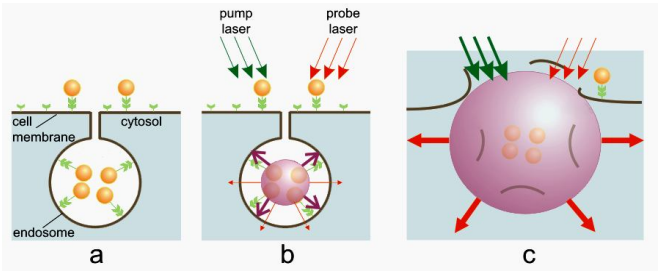

Figure 1: PNB cell theranostic with multi-stage tunable PNB: (f) cell is targeted with NP-antibody conjugates and intracellular NP clusters are formed through the receptor-mediated endocytosis, (b): the 1st (diagnostic) PNB provides the data on a cell and allows to determine the parameters of the next laser pulse, (c) the 2nd PNB delivers mechanical impact (cell damage though membrane disruption is shown) and this action is guided through the increased optical scattering (red arrows) of the 2nd PNB; PNB is tuned by varying the fluence of pump pulse (green arrows).

Optical and mechanical properties of PNB depend upon its diameter (tunable in the range of from 50 nm to 50 μm)

and lifetime (tunable in the range of from 10 ns to 10 μs). Short lifetime of PNBs makes them highly transient phenomena that exist on demand. For target-specific generation of the PNBs we have selectively formed the clusters of relatively safe gold NPs around molecular targets in cancer cells. Gold NPs, conjugated to diagnosis-specific antibodies have been delivered and aggregated into NP clusters through the mechanisms of antibody-antigen interaction and endocytosis (Figure 1(a)). Remote (optical) and non-invasive activation and sensing of PNBs around such intra-cellular clusters was realized in individual living cells with free laser beams.

When activated by a laser pulse, an intracellular plasmonic nanoparticle (Figure 1(b)) act as heat source and generates a transient PNB in the surrounding medium. PNB's of nanometer-scale size and nanosecond-scale duration act as diagnostic probes by scattering light of probe laser. Larger micrometer-scale PNB's provide a localized therapeutic action through a mechanical (non-thermal) impact due to their rapid expansion and collapse, thus disrupting the cell membrane Figure 1(c)). Optical monitoring of the disruptive PNB's can guide their therapeutic action. Thus the PNB's may combine diagnostics, therapy, and therapy guidance.

2.2 Cells

For the *in vitro* experimental model we have used gold spheres of 50 nm and their conjugates with anti-epidermal growth factor receptor (EGFR) antibody C225 that were obtained from Nanopartz Inc (Salt Lake City, UT). The cells were prepared as the monolayers of living EGFR-positive lung carcinoma cells (A549) that were grown into standard 9-mm culture wells (#C24765, Molecular Probes, Inc., Eugene, OR). All cells were incubated with NPs for 30 min at 37°C. The concentration of the NPs during the incubation was adjusted to $0.9*10^{11}$/ml. NP-C225 conjugates were selectively coupled with EGFR. This provided maximal relative concentration of NPs at cellular membrane. Secondly, during receptor-mediated endocytosis the NPs were internalized and concentrated into the clusters of closely packed NPs in endosomal compartments [6,7].

Cell viability was evaluated optically with two standard microscopy techniques. First, a bright field image was obtained for the cell before and after its exposure to a single pump pulse and the difference of these two images was used to detect any PNB-induced changes of the shape of the cell. Second, the membrane damage by PNB was detected using standard fluorescent method by monitoring the cellular uptake of Ethidium Bromide (EtBr) dye that enters only the cells with compromised membrane. Fluorescent images were obtained for each cell before and after PNB generating.

2.3 Optical generation and detection of PNB

PNB generation was experimentally realized by using laser pulse-heated intracellular gold NPs. PNBs were generated around 50 nm gold spheres and in individual living cells. The pulse wavelength (532 nm) and duration (0.5 ns) were chosen so to provide maximal localization of the released heat and at the same time to avoid the generation of shock and pressure waves. If the localization of a photothermal (PT) impact is required, there should be no pressure and shock waves, and also the thermal diffusion losses should be minimized. When the optical pulse duration exceeds acoustic relaxation time, no pressure or shock wave would emerge. Next, when optical pulse duration is less then thermal relaxation time, the losses due to thermal diffusion are negligible, and the entire heat released is concentrated in a small volume around the heat source. In our work we have employed a pulse of length 0.5 ns, 532 nm (STA-01 SH, Standa Ltd, Vilnius). The pump laser beam was directed into the illumination path of an inverted optical microscope and was focused into the sample. Single cells or NPs and single events were studied.

PNB detection has been realized with two optical methods. These methods were applied earlier by us for the imaging of the photothermal phenomena in living cells with the pump-probe laser microscope that we have developed [6-11]. The time-resolved imaging of NPs and PNBs was realized by using side illumination of the sample with a custom made pulsed probe dye laser beam (0.5 ns) at a wavelength 690 nm and with a tunable time delay of 1- 10 ns relative to the pump pulse. The scattered by NP or by PNB probe radiation was imaged with the digital camera (Luka, Andor Technologies, Ireland). While allowing to "see" the PNB the pulsed imaging can hardly provide kinetic measurement. The latter was realized by the thermal lens method in a time response mode. An additional continuous probe beam (633 nm) was directed to the sample collinearly with probe pulse and its axial intensity was monitored by a high-speed photodetector (PDB110AC, Thorlabs Inc.). The time response mode allowed measurement of the PNB lifetime and characterizes a maximal diameter of the bubble and thus allows to quantify its therapeutic impact.

3 RESULTS

3.1 Gold NP-generated PNBs in living cells

Generation and detection of tunable PNBs in living cells was studied in individual living A549 lung carcinoma cells targeted with conjugates of 50 nm gold spheres to anti-epidermal growth factor receptor antibody C225. Optical scattering of the pulsed probe beam (690 nm) by the gold NPs and by the PNBs in the cells was measured as image pixel amplitude (Figure 2-II). Also, the lifetime of the PNB was measured as the duration of a PNB-specific time response that was simultaneously obtained (Figure 2-III). We have monitored the damage to the individual cells after their exposure to the laser pulse by fluorescent imaging of the uptake of the Ethidium Bromide (that stains

cells with disrupted membrane) and the blebbing (that is associated with the cytoskeleton damage). Scattering by gold NPs accumulated by individual A549 cells after 30 min incubation at 37°C (Figure 2a-II) was found to be quite low and its image amplitudes were close to the scattering image amplitudes associated with cellular organelles. We have used NP scattering image as a reference for quantifying the amplification of optical scattering by the PNBs. The first pump laser pulse was applied to individual cells at a fluence of 0.24 J/cm^2 (above the bubble generation threshold), which induced a PNB within the cell as was detected with probe laser image (Figure 2b-II). The lifetime of this PNB was relatively short, 25 ns, according to its time response (Figure 2b-III). This PNB has amplified the scattering by 9.2 times relative to the scattering by the gold NPs. After the PNB generation bright field (Figure 2b-IV) and fluorescent (Figure 2b-V) microscopy images of the cell showed no deviation from the pre-pulse conditions shown in Figure 2a-IV,V, respectively. The absence of fluorescence and blebbing has implied that the cell has survived the laser pulse and the PNB. We have detected only one PNB despite apparent fact that endocytosis assumes the internalization of many NPs. This can be explained with the threshold nature of the PNB: the fluence level was sufficient for the generation of the PNB only around the biggest clusters of NPs, while this fluence was below the PNB generation threshold for the smaller NP clusters or single NPs. This result has demonstrated high specificity of the PNB generation comparing to the specificity of the nanoparticle imaging (Figure 2a-II). The sensitivity of PNB diagnosis versus NP diagnosis is clearly seen by comparing Figure 2a-II with Figure 2b-II: under identical imaging conditions the amplitude of the NP scattering was much lower than that for the PNB scattering so it did not produce any detectible image.

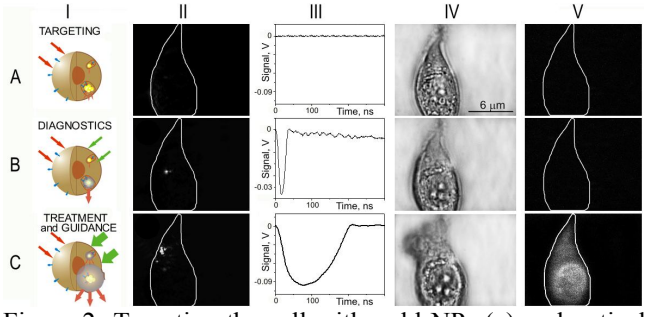

Figure 2: Targeting the cell with gold NPs (a) and optical generation and detection of the intracellular PNBs: the 1st one non-invasively amplifies optical scattering (b), while increasing the fluence of the pump laser pulse induces the 2nd PNB that mechanically damages the cell (c); I – stages of the PNB theranostic action, II- optical pulsed scattering images of one cell with the membrane boarder shown with a white line, III – optical time response of the PNB shows its lifetime, IV - bright light and V - fluorescent (ethidium bromide-specific) images of the cell show it before (a) and after the generation of the 1st (b) and the 2nd (c) PNBs.

Next, the second laser pulse was shortly applied to the same cell at the increased fluence of 1.76 J/cm^2. The second PNB (Figure 2c-II) was much brighter with its scattering amplitude being amplified 290 times relative to that of the NPs and also was much longer (Figure 2c-III) than the 1st PNB. Within 30–60 s after the PNB generation the fluorescent image has shown the penetration of the dye inside the cell (Figure 2c-V) and bright field image has shown the formation of the blebbing bodies (Figure 2c-IV). These have indicated the disruption of the cellular membrane and, possibly, of the cytoskeleton. This experiment has demonstrated the ability to tune the intracellular PNB by varying laser fluence from non-invasive imaging (with an almost 10 fold improvement in optical scattering signal) to cell disruption.

3.2 PNB and cell damage

We have studied the cell damaging properties of the PNB's by varying the laser pulse fluence so to analyze the probability of bubble generation and the probability of cell damage among intact (untreated) and NP-treated cells. Each single cell in the population was irradiated with a single laser pulse of specific fluence and then the cell population-averaged values were obtained (Figure 3). NP treatment has lowered the threshold laser fluence for the bubble generation by almost 30 times relative to the intact cells (Figure 3). As a function of pulse laser fluence, the probabilities of cell damage and of the bubble generation coincided for intact cells, but were significantly separated in the NP-treated cells (Figure 3). At pulse fluences of 0.06–0.22 J/cm^2, intracellular PNBs were generated in NP-treated cells without damaging the majority of the host cells (Figure 3), while the same cells were damaged at 10 times higher fluences.

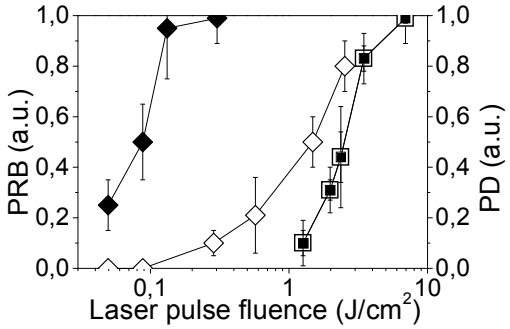

Figure 3: Influence of the fluence of a single pump laser pulse (532 nm, 0.5 ns) on the PNB parameters and on the damage as measured in individual A549 cells: PNB generation probability (PRB): ♦ - cells incubated with NP-C225 conjugates, ■ –intact cells; cell damage probability (PD): ◊ - cells incubated with NP-C225 conjugates, □ – intact cell.

3.3 PNBs as optical probes

Finally, we have evaluated the sensitivity and specificity

of the PNBs as imaging probes. We have measured the optical scattering amplification effect of small (non-invasive) PNBs as a function of the PNB lifetime, since the lifetime correlates with the bubble diameter Figure 4.

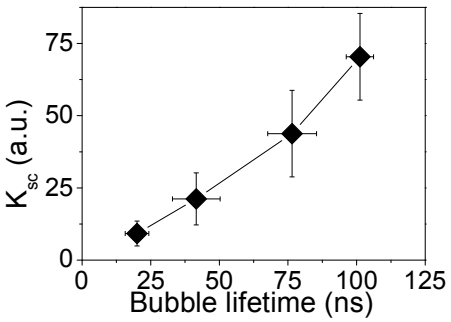

Figure 4: Influence of the fluence of a single pump laser pulse (532 nm, 0.5 ns) on amplification of optical scattering amplitude by the PNB (relatively to gold NPs) in the NP-treated cells as function of the PNB lifetime (i.e maximal size of the PNB).

The data presented were averaged for the cell populations and were obtained at specific laser pulse fluences. An amplification coefficient K_{sc} (measured relative to scattering amplitudes for gold NPs) linearly increases with the PNB lifetime. This implies the PNB-based mechanism of the amplification: the vapor-liquid border of the PNB creates a gradient of the refractive index, and the scattering efficacy of the PNB is determined by its diameter (that correlates to the PNB lifetime). The PNBs have yielded 10 – 50 fold optical amplification without disrupting cell membrane or inducing the blebbing.

Obtained results are the first and laboratory stage proof of the principle for the theranostics with plasmonic nanobubbles. In future PNB can provide a universal platform for basic biomedical research, diagnosis and therapy. Several potential applications of PNBs include (1) high-sensitive non-invasive imaging (based on amplified optical scattering), (2) controlled release, transfection and intracellular delivery (based on localized disruption with the PNBs of specific capsules, endosomes and cellular membrane), and (3) selective and guided cell and tissue damage. Our method was realized with a standard optical microscope. Due to the brief duration of the PNB (nanosecond range) our method can also be realized also in flow cytometry and in micro-fluidic devices. The stealth nature of the PNB, which is not present in the sample until being remotely activated and the safety of gold NPs may provide for their *in vivo* applications. Finally, a real-time tunability of the PNB optical and mechanical properties may allow the combination of different processes in one fast sequence of PNB-supported operations: detecting the target with small non-invasive PNB, destructing the target with a bigger PNB by applying the second laser pulse of increased fluence, and monitoring the destruction though the optical properties of the destructive PNB. Such a theranostic method can be accomplished within a microsecond time scale and can be extended also to molecular or tissue targets. The experimental results presented here have demonstrated the proof of the principle for the PNB theranostic method in individual living cells:

- the ability to use an on-demand activated agent – plasmonic nanobubble – for the diagnostic and therapeutic purposes by tuning its maximal diameter and by using its optical and mechanical properties;
- non-invasive amplification (50 fold relative to the scattering by NPs) of optical scattering by the PNBs in specific cells;
- damage-specific optical parameters of the PNBs (lifetime and optical image amplitude) in disruptive mode provide the basis for the real-time guidance of PNB therapy at cell level.

The development of a universal nontoxic theranostic probe with tunable properties may provide rapid translation of new technologies into clinical phase. Although aimed at cancer, our methods are universal and can be applied to other pathological conditions since the plasmonic nanobubbles can be used at molecular, cellular and tissue levels.

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