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Selective and self-guided micro-ablation of tissue with plasmonic nanobubbles

Ekaterina Y. Lukianova-Hleb, PhD^{a,d}, Irina I. Koneva, PhD^b, Alexander O. Oginsky^a, Saverio La Francesca, MD^c, and Dmitri O. Lapotko, PhD^{a,d}

^aJoint American-Belarusian Laboratory for Fundamental and Biomedical Nanophotonics, Rice University, 6100 Main Street, Houston, TX 77005, USA

^bInstitute of Genetics and Cytology, 27 Academic Street, 200072 Minsk, Belarus

^cDepartment of Cardiovascular Surgery, Methodist DeBakey Heart and Vascular Center, 6550 Fannin, Houston, Texas 77030, USA

^dDepartment of Physics & Astronomy, Rice University, 6100 Main Street, Houston, TX 77005, USA

Abstract

Background—The accuracy, selectivity and safety of surgical and laser methods for tissue elimination are often limited at microscale.

Materials and methods—We developed a novel agent, the plasmonic nanobubble (PNB), for optically guided selective elimination of the target tissue with micrometer precision. PNBs were tested *in vitro* in the two different models of superficial tumors and vascular plaques.

Results—PNBs were selectively generated around gold nanoparticles (delivered to the target tissues) with short laser pulses. Monolayers of cancerous cells and atherosclerotic plaque tissue were eliminated with PNBs with micrometer accuracy and without thermal and mechanical damage to collateral normal tissues. The effect of the PNB was dynamically controlled through the fluence of laser pulses (532 nm, duration 0.5 and 10 ns) and was guided through the optical scattering by PNB.

Conclusions—plasmonic nanobubbles were shown to provide precise, tunable, selective and guided ablation of tissue at a microscopic level and could be employed as a new generation of surgical tools.

Keywords

laser; plasmonic nanobubble; gold nanoparticle; revascularization; superficial tumor

Corresponding author: Dmitri O. Lapotko, PhD, Rice University, Physics and Astronomy - MS 61, 6100 Main Street, Houston, TX 77005, Tel: 713-348-3708, Fax: 713-348-4150, dl5@rice.edu.

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Introduction

The challenges of current surgical techniques include limited selectivity and precision in cases when the target is small (often of micro-size) and is located close to vital organs, as is the case with many superficial tumors¹⁻³ or plaques and thrombi inside coronary vessels.^{4,5} In addition, no surgical tool provides any sort of real-time feedback (guidance), which could be of critical importance in many cases. Laser surgery partially solves the aforementioned problems, but also suffers from low selectivity and safety due to its thermal nature, because it is difficult if not impossible to control the optical and thermal properties of the tissues that act as the optical absorber and as a heat source⁶⁻¹³. As a result, a surgical application cannot be successfully directed to small targets (comparable to cells), thus limiting many of the clinical applications of laser surgery. Some methods of laser surgery do not depend upon tissues properties (like femtosecond pulsed laser surgery¹⁴⁻¹⁸ that works through optical breakdown), but such methods have other, technical limitations that prevent their *in vivo* application.

Nanotechnologies were employed to address the aforementioned problems, but, despite the promise of the properties of nanoparticles, they still often use macroscale, rather than nanoscale processes. This limits the accuracy, efficacy and safety of their medical applications. We hypothesize that by combining the photothermal properties of plasmonic (gold) nanoparticles with the mechanical and optical properties of transient vapor nanobubbles we can produce a tunable surgical micro-tool. This tool is not a nanoparticle (NP) but an NP-generated event, a plasmonic nanobubble (PNB).^{19,20} A PNB emerges when a plasmonic gold NP is locally overheated with a short laser pulse. As a result the NP evaporates a very thin volume (nanometer size) of the surrounding medium, thus creating a vapor nanobubble that expands and then collapses within a short nanosecond. Its fast expansion produces a localized mechanical and non-thermal impact.^{21,22,23} In addition, the bubble scatters the light, thus acting as an optical probe.²⁴ We defined this bubble as a plasmonic nanobubble because a plasmonic NP acts as its source and determines its energy and location. Unlike other vapor bubbles (generated with bulk heating, ultrasound and lasers), the PNB thermally insulates the outer environment from the high temperature of a heated NP,^{21,22} thus reducing the risk of thermal damage to the minimum. Recently we showed that PNBs can be efficiently used at the cellular and tissue level *in vitro* and *in vivo* as a diagnostic, therapeutic, delivery and theranostic agent.^{19,20,23,25-32} Based on the established properties of PNBs, we further hypothesize that plasmonic NPs can be efficiently used as heat sources instead of tissues for micro-surgery in order to achieve micrometer precision and selectivity of tissue elimination. This work evaluates the proof of the principle for using the PNBs for tissue ablation and elimination at micro-scale.

Materials and Methods

Plasmonic nanobubble as surgical agent

A PNB is generated around gold NPs with a short laser pulse and the rapidly expanding bubble mechanically disrupts the surrounding tissue (Figure 1A). Its mechanism is non-thermal since the PNB insulates the surrounding medium from the high temperature of the laser-heated nanoparticle.^{22,33} Furthermore, optical absorbance of gold NPs is 3-6 orders of magnitude higher than that for any molecules. Therefore, a short laser pulse of low fluence heats only the gold NPs and does not influence the tissue. This is the main difference between the PNB method and standard laser surgery: the latter uses the tissue as a heat source while PNBs do not. The maximal size of a PNB, tunable in the range from 100 nm to tens of micrometers (responsible for mechanical impact of PNB) is determined by the fluence of the laser pulse^{32,33} while the PNB generation threshold is determined by the aggregation (clustering) of gold NPs: the bigger the NP cluster, the lower the PNB

generation threshold fluence of the laser pulse.^{21,27} Therefore, PNBs are selectively generated only round the biggest clusters of gold NPs that can be selectively formed in the target cells and tissues.^{27,31,34} Thus the PNB acts as a dynamically tunable on demand ablative agent:

- it can be immediately activated by applying the laser pulse and lasts for nanoseconds;
- the point of PNB action is determined by the location of NP clusters even though a broad laser beam can be applied (since its low fluence will not produce any significant photothermal effect in tissues without NP clusters);
- the size of eliminated tissue is determined by PNB maximal size and location of NP clusters and not by the laser beam size.

An additional important feature of PNBs is their high optical scattering that correlates to PNB size,^{21,28,35} and thus can be used to guide the ablative action of PNBs in real time.

Optical generation and detection of plasmonic nanobubbles

PNB generation was experimentally realized by forming gold NP clusters in cells and at the surface of a plaque segment. We have used 0.5 ns and 10 ns laser pulses at 532 nm (STA-01 SH, Standa Ltd, Vilnius and LS-2132, Lotis TII, Minsk, Belarus) with the beam diameter 20 μm at the target plane for the excitation of gold NPs with an absorbance peak at the above wavelength. Laser beams were directed into the illumination path of an inverted optical microscope Nikon 200 and were focused into the sample (Figure 1B). Samples were treated with single laser pulses or with pulse trains at a frequency of 15 Hz.

PNBs were detected with the two optical methods that were based on the excellent optical scattering properties of PNBs.^{25,26,33} The time-resolved scattering imaging of PNBs was realized by illuminating the sample with an additional laser pulse (0.5 ns, delayed relative to the excitation pulse by 10 ns, wavelength 690 nm and a fluence below 0.1 mJ/cm^2). Light scattered by the PNB was imaged with a digital camera (Luka S, Andor Technologies, Ireland) (Figure 1B). Although allowing us to “see” PNBs, the pulsed scattering imaging method did not provide the kinetic measurements required for monitoring the maximal size of the PNB. The latter was realized with a continuous probe beam (633 nm) that was directed at the sample collinearly with the probe pulse and its axial intensity was monitored by a high-speed photodetector (PDA110AC, Thorlabs Inc., Newton, NJ). This allowed us to detect a PNB-specific time response, the duration of which was proportional to the maximal size of PNB.^{21,33} The image and response modes were applied simultaneously, thus combining the imaging and quantitative studies of PNBs.

Tissue samples

We employed two *in vitro* models. A cell monolayer model was studied the first in order to evaluate the PNBs for superficial targets such as tumors. Next, a tissue model was studied to evaluate the PNBs for more challenging tissues, such as plaques in coronary vessels. The monolayers of living cells modelled a superficial tumor and were prepared from epidermal growth factor receptor (EGFR)-positive lung carcinoma cells (A549) and EGFR-negative normal cells, fibroblasts. The cells were grown to the confluence of 98% in standard 96-wells culture plates. The period of growth was 24-30 hours at 37°C in a CO₂ incubator. For targeting we used gold spheres of 60 nm conjugated with anti-EGFR antibody C225 (BioAssay Works L.L.C., Ijamsville, MD). All cells were incubated with NPs for 30 minutes at 37°C in the CO₂ incubator. This incubation procedure provided the formation of large NP-clusters selectively in EGFR-positive cells with the mechanism of receptor-mediated

endocytosis.^{27,28,32,34} After the clusters are formed, the fluence of laser pulse can be reduced so as to generate PNBs only around NP clusters and not around single NPs.

The tissue model represented human postmortem artery slices of stenosed atherosclerotic plaques. This model was used to evaluate an efficacy and accuracy of tissue ablation with PNB under confined conditions and when high precision and selectivity of tissue elimination are required. In cases when (1) plaque totally blocks the artery, (2) plaque is so hard that it cannot be penetrated by a standard wire to guide the stent into the plaque and (3) this part of the artery cannot be removed or shunted the ablative effect of plasmonic nanobubbles may at least to help in drilling small channel through the plaque that will allow for the wiring of a catheter guide. Histological slices with a thickness of 20 μm were prepared by the standard method on cryomicrotome setup³⁶ from the segments of the postmortem coronary human arteries. In our study we used only totally occluded arteries with calcified plaque that included stone-hard segments. We pre-treated slices with NP clusters due to the absence of plaque specific NP-conjugates. In this case we used aqueous suspensions of gold spheres with diameters of 50, 90 and 250 nm (TedPella Inc, Redding, CA). NP clusters were prepared by adding PBS to the aqueous suspensions of NPs in a 1:1 ratio. We used a static model of treatment with NPs: a single slice placed in a microcuvette which was completely filled with suspension of NP clusters and sealed by a cover slip. After 30 minutes, all NP-clusters were deposited on the slice surface and the sample was used for PNB generation. For control we used untreated plaque slices.

Quantitative parameters

PNB lifetime (τ)—Characterizes the mechanical impact of PNB and was measured (at the 0.5 level of the maximal amplitude of the response) through the maximal diameter of the PNB.³⁷ This parameter was measured as the duration of PNB time response.

Probability of PNB generation (PRB)—Was introduced to characterize the conditions of PNB generation and was obtained statistically after observation of N events:

$$PRB = \frac{N}{N_1},$$

where N = the amount of PNB-specific responses in irradiated objects, and N_1 = the amount of irradiated objects. To determine the PNB generation threshold, values of PRB were obtained at several pulse fluencies, so that the one corresponding to PRB level of 0.5 was defined as the *PNB generation threshold* ($\varepsilon(0.5)$) for a given sample. PNB generation probability PRB was measured at several pump pulse fluence levels so as to cover the range from those close to 0 (no PNBs) to those close to 1 (the PNBs were generated in 100% of the cells). As the laser fluence increased, the PRB values gradually increased from 0 to 1 and the monotonous character of the $PRB(\varepsilon)$ dependence was found to be typical for all studied cells and NPs.

The cell damage level (LD)—Was measured after laser treatment, with the 0.4% trypan blue dye, then washed and imaged so as to count the non-stained (living) and stained (dead) cells in irradiated and intact areas. The cell damage level for each sample was determined as:

$$LD = 1 - \frac{M_2}{M_1}$$

where M_1 is the amount of living cells in non-irradiated areas, and M_2 is the amount of living cells in irradiated areas. So $LD = 1$ means 100% damage, and $LD = 0$ means no damage after laser irradiation. Ten identical samples were studied.

Recanalization coefficient (K_{rec})—Was introduced as a metric of the efficacy of tissue elimination (Figure 2). We have used a continuous probe laser beam (633 nm) in order to guide the elimination process. The relative level of the intensity of the probe laser I as measured at the exit from the sample depends upon the thickness H of the sample. As the PNB eliminates the tissue to the depth h the transparency of the sample increases, thus increasing the probe laser signal I from the initial minimal level I_0 to the maximal level I_{max} that corresponds to the tissue-free optical path:

$$K_{rec} = \frac{h}{H} = \frac{I - I_0}{I_{max} - I_0}.$$

This parameter was obtained by measuring I_{max} for the tissue-free zone, I_0 for the tissue before generating the PNBs and I after each PNB. Partial elimination of the sample would correspond to $0 < K_{rec} < 1$, the absence of any effect would correspond to $K_{rec} = 0$. This parameter is directly proportional to the percentage of the removed tissue within the aperture of the probe (guiding) laser beam. For example, the value of $K_{rec} = 0.5$ means that 50% of the plaque tissue has been removed. This coefficient was especially helpful when plasmonic nanobubbles did not totally remove the tissue, since under optical observation it is difficult to differentiate the partial removal of the tissue from the intact one. However, with this coefficient we were able to monitor the progress of tissue elimination even when it was not apparently visible and we were able to describe the process of tissue ablation in a quantitative way and to further correlate this process to the quantitative parameters of PNBs, gold NPs and laser radiation.

Results

Elimination of monolayers of cells

The monolayers of cancer (A549) and normal (fibroblasts) cells were scanned with the pump laser pulses in the pattern showed in Figure 3A. The size of the treated area was $240 \times 120 \mu\text{m}$.

Firstly, we applied single laser pulses to the samples of cancer and normal cells and so determined the PNB generation threshold fluences for single 0.5 ns pulses (Table 1). PNBs were identified according to their specific time responses (Figure 4A,B) of negative and symmetrical shape that is specific for PNB³⁷. The cell population-averaged PNB parameters (PNB lifetime and the generation threshold laser fluences) were obtained for each sample. The threshold of PNB generation in A549 cells treated with NPs was almost 4 times lower than in identically NP-treated fibroblasts, and more than 37 times lower than in intact cells that were not treated with gold NPs (Table 1).

The lifetimes (and hence the maximal diameters³⁷) of the PNBs generated in cancer and normal NP-treated cells were found to be very different at the same level of laser fluence

and after identical treatment with gold NP conjugates (Figure 4A,B): the PNBs observed in cancerous cells were almost four times bigger than those in non-cancerous cells. Next, we scanned the laser pulses (532 nm, 0.5 ns) across the sample (Figure 3A) at the pulse frequency of 15 Hz with the laser pulse fluence 116 mJ/cm² and analyzed the cell damage and elimination. In this scan mode each cell in the layer received a single laser pulse. PNB lifetimes were recorded simultaneously during the scan and differed significantly for cancer and normal cells (Figure 5A). None (or only relatively small ones) PNBs were detected in intact cells under identical conditions. Based on our previous results²⁸ we consider that only the PNBs with a lifetime above 110 ns damaged the cells. This was confirmed during the viability checks with Trypan Blue staining: only cancer cells were damaged and therefore, stained blue (Figure 3B) while normal cells were not (Figure 3C).

By varying the laser pulse fluence (and hence the PNB lifetime) we achieved almost 100% damage to cancer cells (Figure 3B, 5B) without damaging normal cells (Figure 3C). The damage area for cancer cells (Figure 3B) coincided with the scan area (Figure 3A) and the cells outside the scan area were not damaged. This demonstrated the localized and cell-selective action of PNBs. At the fluence level applied, the PNBs only damaged but did not remove the cells so it was not full elimination mode.

To achieve the complete elimination of the cell monolayer we increased the pump laser fluence to 220 mJ/cm² (that was still below the PNB generation threshold fluence for normal cells, as shown in Table 1). After a single scan we observed the cell elimination (removal) only in the scanned area (Figure 3D). PNB lifetime (and hence their maximal size and mechanical impact) in this “ablative” mode increased up to 300-500 ns (from 170 ns for the “therapeutic” mode). During the generation of these big PNBs we observed no damage to the normal cells and no PNBs (or small ones only) were detected in normal cells (with the lifetime below 110 ns, which is considered to be the damage threshold for cells²⁸). Therefore, the PNB method of cell elimination was very selective and at the same time provided micrometer accuracy of cell elimination.

Analysis of shapes and amplitudes of all obtained PNB responses (for cancer cells) has revealed that the temperature of the medium outside the PNB did not deviate from an ambient level after the collapse of the PNB, since the signal precisely returned to its baseline (Figure 4A). This result is in line with our recent data on the thermal insulating effect of PNB's^{22,33} (that proved that, unlike laser-heated NPs, PNBs do not deliver significant thermal impact to their environment). Based on the results obtained we concluded that PNBs can be used in two modes: therapeutic (target cells are selectively damaged, but are not removed), and ablative (the layer of the target cells is removed by PNBs) and the selectivity and precision of the target elimination was within 10 µm. Cell elimination was achieved in with single pulses and after one scan and did not require prolonged exposure of the tissue to laser radiation.

Elimination of atherosclerotic plaque

In this model PNBs were generated in arterial slices around NP clusters. The bright field images of the plaque tissue were obtained before and after the PNB treatment in scan mode (Figure 6). We have applied a longer, 10-ns laser pulses and bigger, 250 nm gold NPs in order to provide larger PNBs with a stronger mechanical impact. After a single scan of the NP-treated plaque we have achieved full elimination of the plaque tissue (Figure 6B). This “cold” effect of total elimination of the plaque was achieved without any detectable thermal impact on surrounding tissue. After verifying the effect of PNBs in a plaque we studied in detail the mechanism of tissue elimination. We have measured the recanalization coefficient (which was 1.0 for the case presented in the Figure 6B) as a function of PNB and laser parameters. For better accuracy of quantitative measurements we initially calibrated K_{rec} for

the specific samples. The intensity of a continuous probe laser beam was measured after passing through the slices of different thicknesses from 0 to 50 μm (Figure 7A): the thicker the tissue, the lower the intensity of probe laser passing through the slice. For the samples of different thicknesses the recanalization coefficient was calculated by assuming that the initial thickness H of a slice is equal to 50 μm and $K_{rec}=0$, and in the case of the absence of tissue ($H=0$) $K_{rec}=1$. We have found that this coefficient is almost linearly proportional to the thickness of the sample (Figure 7A). Based on the known cross-section of the probe laser beam we have further correlated K_{rec} to the volume of the removed tissue (Figure 7B). Since the value of K_{rec} can be obtained continuously during multiple scans, it allowed us to monitor the volume of the eliminated tissue for the sample with specific thicknesses.

The plaque samples with a thickness of 20 μm were treated by an NP cluster suspension and then several different regions were irradiated by single laser pulses at the different laser pulse fluencies from 1 to 5 J/cm^2 (15 regions in 3 samples were analyzed for each level of laser fluence). PNB lifetime and K_{rec} were monitored simultaneously after each laser pulse. We observed a good correlation between the recanalization coefficient and the volume of the removed tissue (Figure 7B). At the same time K_{rec} correlated perfectly to the PNB lifetime for the same samples (Figure 7C). Therefore, we concluded that the tissue elimination in this experiment was provided by the generation of PNBs and the volume of the eliminated tissue was determined by the PNB size. We have also found that a single pulse mode did not provide full elimination of the tissue, since the values of the K_{rec} were below 1.0 (Figure 7C). To improve the effect of PNBs we applied the pulse trains instead of single pulses and analyzed the K_{rec} as the function of the laser pulse fluence and of the number of laser pulses in train (Figure 7D). Each individual zone of the plaque was irradiated by 1 to 10 laser pulses at specific laser pulse fluence (1.5, 3 and 5 J/cm^2). We observed that each subsequent pulse increased the recanalization coefficient from 0.24 after the first pulse and to 0.85 after the tens pulse (Figure 7D). The pulse fluence also contributed to the ablative effect of PNBs (Figure 7D). Therefore, the tissue elimination mechanism had a cumulative nature even though there was no continuous delivery of new gold NPs to the treatment area. Under the same treatment conditions the recanalization coefficient for the artery wall was found to be close to zero (Figure 7D), and no PNBs were observed in this zone. Therefore, the generation of PNBs around clusters of gold NPs was selectively associated with the plaque tissue and did not affect the artery wall though the latter has been exposed to the excitation laser radiation.

Finally, we investigated the dependence of the K_{rec} upon the diameter of the gold NPs. The plaque samples were treated with the gold spheres of 50, 90 and 250 nm diameter at the same concentration of NPs ($3 \cdot 10^{10}/\text{ml}$). PNBs were generated by pulse trains of 40 second duration. The maximal ablative effect was achieved with the biggest NPs of 250 nm (Figure 7E). These experiments demonstrated the tunability and selectivity of PNB effect on tissue elimination in atherosclerotic plaques. It was important that the tissue was not a part of the mechanism of the PNB generation and the parameters of the PNBs depended only upon the parameters of gold NPs and laser pulses. This property of the PNB method improves its precision compared to other laser methods where the eliminating effect depends upon the photothermal properties of the tissue.

Optical guidance of PNB action

In real tissues the threshold of the PNB generation and PNB size significantly depend upon local conditions that influence the laser fluence and the size of NP clusters. Thus, a realtime guidance of PNB generation would help to control its ablative efficacy and safety *in vivo*. The guidance potential of the PNB method was analyzed for both the models. In the monolayer model we used PNB time responses and observed that PNB lifetime correlated to the cell damage (Figure 5B). Based on the obtained results we estimated the cell damage

threshold lifetime of the PNB to be about 110 ± 12 ns and this matches our previous results obtained for other samples.^{28,31} Therefore, PNB lifetime can be used for quantitative and realtime optical guidance of the ablative effect of the PNB. In the case of the plaque model, we used PNB responses and scattering images (Figure 8).

During the irradiation of the plaque sample with the pump laser pulses, PNB time-responses (Figure 4C) and the time-resolved scattering images (Figure 8B) were detected simultaneously. PNBs were characterized by relatively high (compared to the surrounding tissue and NP clusters) pixel image amplitude (6-10 times higher than that for NP clusters, shown at Figure 8A). High-amplitude signals in scattering images (Figure 8D) spatially coincided with the locations of the cleared zones. The duration of time response characterized the lifetime of PNB that is proportional to its maximal diameter and thus describes the PNB mechanical impact. Also the shape of the time responses (Figure 4C), indicated the absence of any substantial residual heating around and after the collapse of the PNB (as in the case of cells layers). Based on the previous results the lifetime of the PNB also correlated to the volume of the eliminated tissue (Figure 7C). Therefore, real-time optical scattering signals obtained from PNBs during their application can characterize their ablative effect and may help to control the PNB effect through the fluence of the excitation laser pulse. This unique combination of ablative and guidance features makes PNB very promising candidates for the surgical applications where microscale and precision are important. For example, a real time optical guidance of the PNB can be very helpful for optimizing the PNB size in a heterogeneous tissue, which properties vary from point to point. We have used the microscope to register optical signals of PNBs. However, in real conditions, optical fibers (similar to those employed in modern laser catheters for intravascular delivery of laser radiation^{38,39}) can also serve to register images.

Discussion

The mechanism of tissue elimination and ablation with PNB

PNBs demonstrated their ability to mechanically eliminate the tissue, and at the same time to guide optically its effect in real time, in a quantitative way (using PNB optical scattering amplitude and PNB lifetimes). Unlike other vapor bubbles (generated with heat, ultrasound and optical breakdown)^{10,11,13-15,40-44}, the PNB thermally insulates the outer environment from the high temperature of a heated NP, thus reducing the risk of thermal damage to a minimum. The mechanical and optical properties of the PNB are determined by its maximal diameter. This in turn depends upon optical energy being absorbed and converted into heat by the plasmonic NP. For these reasons, PNB properties can be precisely varied or tuned with the energy of a laser pulse (from 100 nm to tens of micrometers). Since the generation of a PNB occurs at the location of the NP, specific targeting of NPs will provide a very specific and localized effect of PNBs. According to our results, shorter laser pulses can provide similar surgical efficacy at much lower fluence: for 10 ns pulses we observed surgical effects of PNBs at the fluence of $1-5 \text{ J/cm}^2$ while for 0.5 ns pulses the similar effects were observed at 0.2 J/cm^2 . Based on our result we may point out several features of PNBs as potential surgical micro-tools:

- the efficacy of tissue elimination increased with the PNB lifetime and with the number of PNBs generated at one site.
- the tissue elimination effect (as the size of the cleared zone and as the rec. coefficient) increased with the size of the gold NPs and as a result of their clusterization;

- tissue elimination took place selectively only in the locations of NP clusters, and was not observed in the intact cells or tissues under identical parameters of laser treatment.

This mechanism of tissue elimination has a non-thermal, mechanical, disruptive nature due to the PNBs and allows for broad laser beams without precise focusing on a target.

PNB versus current surgical methods and surgical potential of PNBs

The proposed PNB-based method differs from a laser surgery in thermal (long pulses and c. w. radiation) and optical breakdown (ultra-short pulses) modes, in which tissue absorbs optical energy and acts as a heat source.^{6-8,10-18} The PNB-based method also differs from ultrasound surgery, which uses gas-filled microparticles for thrombolytic and atherosclerotic therapies.^{45,46} It is much easier to deliver and to target the smaller and safer gold NPs (systemically or locally) compared to the relatively large and less stable microparticles. Also, ultrasound mechanisms cannot provide the spatial selectivity of PNBs as was demonstrated in our experiments. The main advantages of PNB method are the ability to control, tune and guide PNBs, and the selective and non-thermal nature of tissue elimination. This was not the case with laser-induced bubbles already used in laser angioplasty, where the generation mechanism caused their unregulated growth and damaged artery walls.¹⁰⁻¹³

The prospective surgical applications of this technology reside in the nanoscale selectivity, tunability and guidance of the PNBs effect. Gold NPs can be functionalized with target-specific ligands to selectively bind target tissues.^{48,49} In our work, the PNBs effect was achieved independently from the specific composition of the heterogeneous plaque samples. Real plaques include dense and cellular fibrous tissue, foam cells and calcified tissue.⁴⁹ It is of paramount significance that the debulking effect was observed in all cases. The PNBs could be employed in the treatment of many different atherosclerotic lesions *in vivo*: from heavily calcified lesions to acute occlusions where the presence of thrombi is quite common. Considering hybrid procedures, this technology could be implemented concomitantly with surgical coronary grafting to reach areas not amenable by surgical treatment, in order to achieve a more complete revascularization.

Translation of PNB method to clinical applications requires the generation and detection of PNBs *in vivo*. The safety of gold NPs, the local mechanical action of the PNBs at cell level, the short treatment time and combination of the diagnosis, ablation and guidance in one procedure, will increase the efficacy and safety of PNB in surgical applications. The technical solution for this is a fiber optic system for the remote generation and detection of PNBs that is currently under development in our lab (Figure 9). The fiber optic probe will deliver to the target site the NPs, optically transparent fluid and the laser radiation, and will collect the light backscattered by PNBs. This fiber probe can be connected to the pump and probe lasers and to the syringe pumps with NP suspension and with optically transparent buffer fluid. NP delivery to the target can be realized through topical injection through this probe into the specific area. PNB generation in tissue can be performed within 100 – 200 μm of the distal tip of the probe; PNB detection in a response mode with the light scattered by PNBs being collected by the fiber probe allows real-time guidance of the PNBs. Instead of visible laser radiation (we have used 532 nm pulses) we plan to apply near-infrared wavelength that have better penetration range in the tissues and are safer. Gold nanoshells and nanorods can be used in this case instead of the spheres to match the plasmon resonance wavelength in the near-infrared. This system could also be combined with robotic arm to precisely control the fiber probe.⁵⁰ We expect that PNB fiber optic ablation can improve the outcome of surgical treatment of superficial tumors, intravascular targets and, in addition, can be considered as an alternative for such methods as brachytherapy, because it will allow

the replacement of dangerous radioactivity and complex MRI monitoring with an all-in-one PNB system.

Conclusions

We demonstrated that PNBs can be used as a micro-ablative agent that provided:

1. The reduced to 100-200 mJ/cm² fluence of laser pulse (due to high photothermal efficacy of NP-cluster-PNB mechanism).
2. The localized, selective and tunable mechanical impact on a tissue with the point of effort being determined by the location of NP clusters and not by the whole laser beam.
3. Low or none thermal collateral damage to normal tissues.
4. Optical quantitative guidance of the tissue elimination.
5. Controllable and precise removal of the tissues with the accuracy of about 10 μ m.

The result of PNB ablation was determined by the parameters of gold nanoparticles and laser pulses and did not depend upon photothermal properties of the tissue since it was excluded from the mechanism of PNB generation. Our results suggest that PNB can be potentially employed as micro-surgical tools.

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Figure 1.

(A): The stages of tissue elimination with PNBs (from top to bottom): 1) targeting the tissues with of gold nanoparticles (NP), 2) optical activation of gold NPs with a short pump laser pulse; 3) generation of PNB and its monitoring through the optical scattering of the probe laser beam, 4) elimination of tissue due to mechanical impact of PNB; (b) the microscope-based experimental set up for a PNB treatment of tissue samples: 0.5 ns and 10 ns pulsed pump laser for PNB generation; a pulsed probe laser for time-resolved optical scattering imaging of PNB; continuous probe laser for the monitoring the PNBs time-response and for measuring the recanalization coefficient.

Figure 2.

Principle of a recanalization coefficient: (a) – the initial level I_0 of the pass-through intensity of the probe laser radiation, (b) - elimination the tissue for the depth h increases the laser intensity I , (c) - total elimination of the tissue corresponds to the maximum laser intensity I_{max} .

Figure 3. PNB in cells layers: (a) laser scan scheme; (b) cell damaging mode for A549 and (c) fibroblast cells (blue – dead cells, yellow – live cells; laser: 532 nm, 0.5 ns, 116 mJ/cm²); (d) cell elimination mode for A549 cells (laser: 532 nm, 0.5 ns, 220 mJ/cm²).

Figure 4.

PNB time responses for: (a) NP- treated layer of cancerous (A549) cells, (b) NP-treated layer of normal (fibroblast) cells (laser pulse: 532 nm, 0.5 ns, 90 mJ/cm²); (c) NP-treated and atherosclerotic plaque tissue sample, (d) intact atherosclerotic plaque tissue sample (laser pulse: 532 nm, 10 ns, 3 J/cm²).

Figure 5.

(A) – dependence of the PNB lifetime upon pump laser pulse fluence for cancer (■ – NP-treated, □ – NP untreated) and normal (● – NP-treated, ○ – NP untreated) cells (laser pulse: 532 nm, 0.5 ns); (b) – dependence of cell damage level upon PNB lifetime for NP-treated A549 (cancer) cells (laser: 532 nm, 0.5 ns).

Figure 6.

The bright field microscopy images of the gold NP-treated atherosclerotic plaque tissue sample before (a) and after (b) scanning its surface with multiple 10-ns laser pulses that caused generation of PNBs. Dashed green line shows the scan area with the cleared area (glass bottom surface) inside. Scale bar is 20 μm .

Figure 7.

Dependence of probe laser intensity ($\square$) and the recanalization coefficient ($\blacksquare$) as function of: (a) thickness of the slice, (b) eliminated volume (laser: 532 nm, 10 ns), (c) PNB lifetime (laser 532 nm, 10 ns), (e) NP diameter in multi-pulse mode (laser: 532 nm, 10 ns, 3 J/cm²) and (d) number of pump laser pulses ($\blacksquare$ – laser pulse fluence 5 J/cm², $\blacktriangle$ – 3 J/cm², $\blacktriangledown$ – 1,5 J/cm², $\circ$ - intact tissue @ 1.5 J/cm², laser: 532 nm, 10 ns).

Figure 8. Optical scattering images of: (a) plaque tissue after treatment with gold NP clusters, (b) – PNBs in the plaque sample (laser: 532 nm, 10 ns, 3 J/cm²) and (c-d) are the corresponding pixel amplitude profiles. Scale bar is 8 μ m.

Figure 9.

PNB system for *in vivo* surgical applications: fiber optic probe (catheter) is connected to the pump and probe lasers and to the syringe pumps with NP suspension and with optically transparent buffer fluid and has 4 channels: optical delivery, optical collection, NP suspension delivery, transparent buffer delivery; geometry of the probe provides local generation PNBs; the PNB detection in a response mode is used for a realtime guidance of the PNBs.

Table 1
PNB generation threshold in cells

Tissue sample	Bubble generation threshold, mJ/cm ²
NP-treated layer of A549 (cancer) cells	67±2
Layer of intact A549 cells	2500
NP-treated layer of fibroblast cells	266±22
Layer of intact fibroblast cells	> 3000