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Medical Laser Application

High Precision Cell Surgery with Nanoparticles?

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Summary

Irradiation of nanoabsorbers with pico- and nanosecond laser pulses could result in thermal effects with a spatial confinement of less than 50 nm. Therefore absorbing nanoparticles may be used for creating controlled intracellular effects.

Conjugates of colloidal gold and alkaline phosphatase (aP) are presented as a model system for investigating protein inactivation in the vicinity of strongly absorbing nanoparticles. aP was coupled either directly or via antibodies to 15 nm gold particles. These conjugates were irradiated with picosecond pulses emitted by a frequency doubled Nd:YLF laser. Denaturation was detected as a loss of protein function with the help of a fluorescence assay.

Irradiation with 10^4 pulses resulted in the inactivation of aP at an irradiance of 50 mJ/cm^2 per pulse when the protein was coupled directly to the particles. With similar irradiation parameters a significant inactivation of aP which was coupled not directly but via two antibodies to the gold, was not observed. This shows that inactivating proteins with nanoabsorbers under irradiation with picosecond laser pulses is possible with a high spatial confinement.

However, from these experiments it is difficult to determine whether the aP was destroyed thermally or whether it was inactivated by a photochemical reaction. Therefore, further experiments are needed to elucidate the damage mechanisms.

Key words
Selective photothermolysis, protein denaturation, nanoparticles, colloidal gold

Introduction

Lasers can produce extremely precise and selective effects in biological tissue. Ablation of sub-micron layers of cornea (17), cutting of microscopic structures (10, 13) and the selective destruction of pigmented cell layers (15) have been demonstrated. In recent years, significant progress towards even more precise and more selective diagnostic and therapeutic laser applications has been observed, but the modification of subcellular structures in living tissue and cells is still a great challenge.

In principle, there are two approaches to creating laser effects with subcellular precision. The laser beam can either be focused on a small spot, which is directed to a certain region, or a higher absorption in the tissue can be used for selectively transferring energy to the target (Fig. 1). High precision cell surgery by a focused beam requires high quality optics to create a submicrometer laser spot and knowledge of the spatial localisation of the target to which the laser beam has to be directed. Using highly absorbing molecules or particles, which can be brought to the target structure by antibodies or other target specific molecules, irradiation with an extended beam using simple optics is possible and it is not necessary to know the position of the target. Therefore the second approach is especially suitable for targeting subcellular structures in tissue. The destruction of the target is brought about by photochemical reactions, as used in photodynamic therapy (4) or CALI (9, 11), or by mechanical or thermal mechanisms. A major disadvantage of photochemical reactions is the dependence of their efficacy and damage range on the chemical environment. In contrast,

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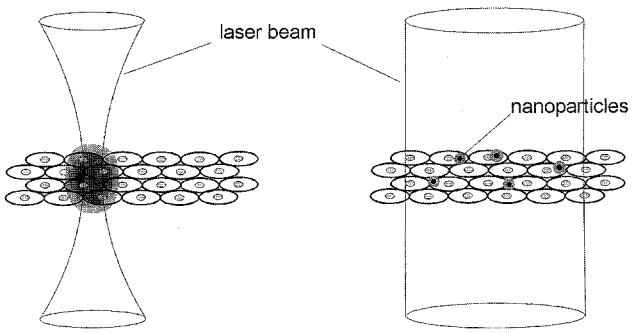

Fig. 1. Principle of high precision laser surgery by using a focused beam (left) or the increased absorption of nanoparticles in the target region (right).

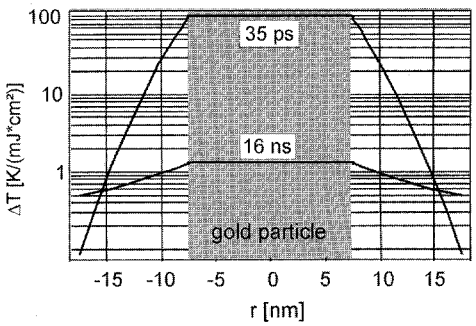

Fig. 2. Spatial temperature distribution around a 15 nm gold particle after irradiation with 1 mJ/cm² at a wavelength of 530 nm. Temperatures were calculated at the end of pulses with 35 ps and 16 ns pulse widths.

physical mechanisms are mostly independent of the specific target structure. Additionally, they can be influenced by the irradiation parameters. Therefore, the objective of this work was to investigate whether thermal damage with subcellular precision is possible by using highly absorbing nanoparticles.

Theoretical considerations

Localised thermal damage is only possible, if the thermal diffusion to the surroundings of the target is reduced by using sufficiently short irradiation times. Anderson and Parrish (1) showed that thermal damage can be confined to the absorbing structures by using laser pulses shorter than its thermal relaxation time

$$t_{th} = \frac{d^2}{27\alpha},$$

which depends on the thermal diffusivity α and the characteristic diameter d of the structure. For targeting larger tissue structures like blood vessels, hair follicles or cells of the retinal pigment epithelium, milli- and microsecond laser pulses are already used in clinical applications (3, 5, 14), targeting subcellular structures requires an irradiation with nano- or even picosecond laser pulses. Since thermal damage depends on time and temperature according to the Arrhenius equation (2, 12), temperatures of 400 K or higher are needed when these short laser pulses are used (7). Calculations (8) show that these high temperatures can be easily reached on the surface of gold nanoparticles which have an extremely high absorption coefficient (Fig. 2). With picosecond pulses temperatures more than thousand Kelvin can be created in a volume of only 5×10^{-21} liter. In order to investigate whether thermal protein denaturation is possible on a submicrosecond time scale, a model system consisting of the protein alkaline phosphatase (aP) coupled to 15 nm gold particles was developed. The proteins were either immobilized directly on the surface of the particles, or bound to antibodies with which the parti-

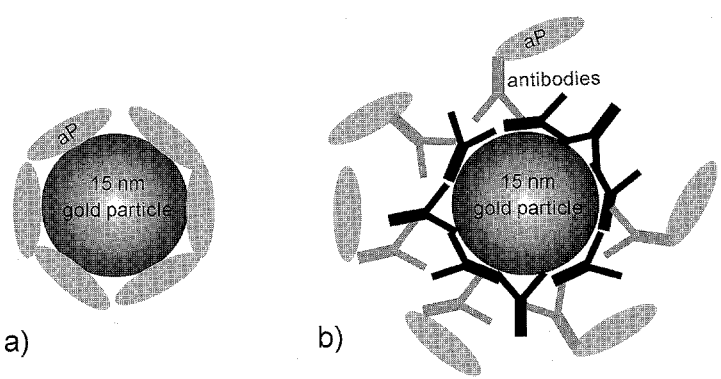

Fig. 3. Schematic picture of the aP-gold conjugates which were used in the experiments. a) directly coupled aP. b) via antibodies indirectly coupled aP.

cles had been coated (Fig. 3). By this indirect coupling, the phosphatase was held at a distance of at least the diameter of one antibody from the particle surface. The particles were irradiated with nano- and picosecond laser pulses and the enzymatic activity of aP was measured by a fluorometric assay before and after irradiation.

Material and Methods

Conjugates were made of 15 nm gold particles, which were purchased from British Biocell International (BBIGC15), and bovine intestinal alkaline phosphatase (Sigma, p5521) according to the usual protocols of antibody gold conjugation (6). For the indirect coupling of aP, the colloidal gold was coated with a mouse anti-human antibody (Dako, M7240). These primary gold conjugates were washed twice by centrifugation at 15000 g for one hour and were then resuspended in TRIS buffer at pH 8. Afterwards these conjugates were incubated with alkaline phosphatase which was covalently bound to a goat anti-mouse antibody (Dako D0486). The gold suspension was again washed twice by centrifugation as described above and the loose pellet consisting of complexes of gold, primary antibody, secondary antibody and alkaline phosphatase was resuspended in TRIS buffer at pH 8. At the beginning of each experiment unbound proteins were removed by centrifugation at 15000 g for 45 min, because a certain amount of protein separates from the gold particles during storage. The loose pellet was carefully transferred to a new vial and resuspended in 50 mM phosphate buffer (pH 7.6).

The fluorescent substrate 4-MUP (4-Methylumbelliferyl-phosphate, Calbiochem) was used to measure the activity of alkaline phosphatase at pH 9.8 with a self-built fluorescence detector. Samples were excited with a Xenon-arc lamp which was equipped with a bandpass at 370 nm. Changes in the emission of the fluorescence substrate were measured at 445 nm. A conversion of 4-MUP to 4-MU through the catalytic action of aP results in an increase in the measured fluorescence signal at this wavelength.

A mode-locked frequency doubled Nd:YLF laser (ISL 2001 MPL, Intelligent Laser Systems Inc.) which generates 35 ps pulses at 527 nm with an energy of up to 300 μJ at a rate of 1 kHz was used to irradiate the conjugates (8). The laser had a stable output energy (5% standard deviation) and a Gaussian intensity distribution.

The aP-gold conjugates were irradiated in wells with a diameter of 2 mm which were custom-made in a 25 mm × 75 mm slide of optical glass (Hellma). The glass slide was positioned under a microscope, the illumination system of which was replaced by a dichroic mirror in order to guide the irradiation beam to the sample and separate frequency-doubled light from the infrared irradiation. For the irradiation, the unexpanded laser beam, which had a diameter of approximately 0.5 mm, was scanned in a field of 4 × 4 spots over the sample area. A nearly homogeneous total radiant exposure of up to 50 mJ/cm^2 per pulse was achieved. In order to achieve higher irradiances the aP-antibody-gold conjugates were spotted onto a glass plate, forming droplets with a diameter of 500 μm. Each droplet was scanned with a Gaussian beam at 4 × 4 locations.

Result

It was shown that the fluorescence assay can quantify the aP activity, if the concentrations of aP and 4-MUP were chosen in an appropriate range, in which the slope of the increase of the 4-MUP fluorescence is proportional to the aP activity (Fig. 4). When immobilized to the surface of the gold particle, aP still showed enzymatic activity which could be measured even in small samples of a few micro liters. Irradiation of conjugates with single pulses at 50 mJ/cm^2, which was the maximal possible irradiance in these experiments, had no observable effect. Since thermal and photochemical effects of different pulses are additive, multiple pulses were directed to the samples. At an irradiance of less than 5 mJ/cm^2 even with 10^4 pulses no effect was observed. But when the irradiance was raised to 50 mJ/cm^2 the activity of the bound proteins was reduced to 25%. Irradiating aP, which was not bound to gold nanoparticles with these laser parameters, only a slight reduction of the protein activity was observed. When the conjugates in which aP was coupled to the gold particles via two antibodies were irradiated the effects were comparable to the irradiation of unbound proteins.

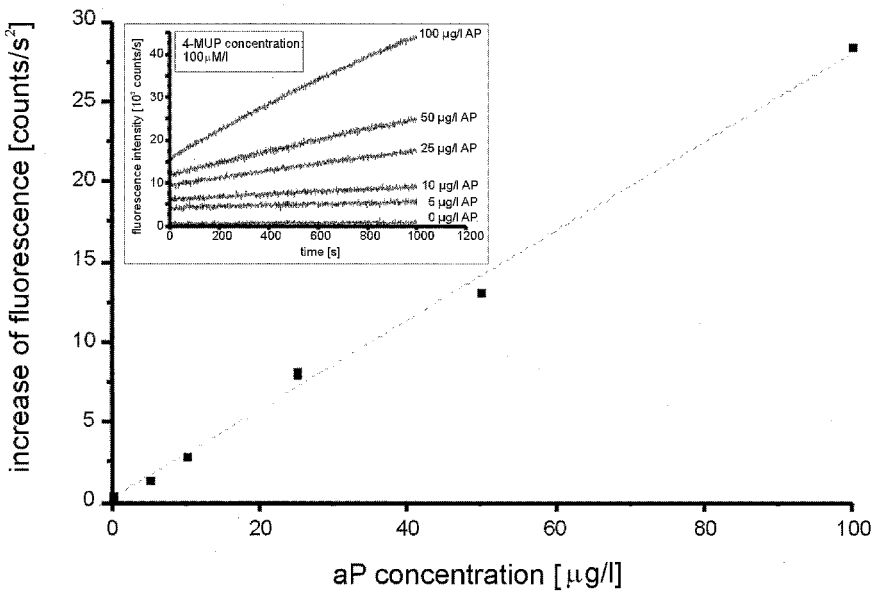

Fig. 4. Linearity of the fluorescence assay. The slope of the increase of substrate fluoresecence was plotted versus the concentration of alkaline phosphatase. The inset shows the fluorescence, which is caused over time by the enzymatic conversion of 4-MUP to 4-MU for the different aP concentrations.

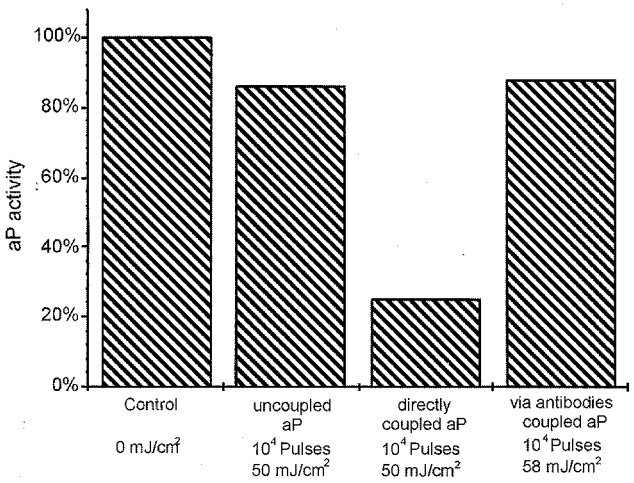

Fig. 5. Activity of aP in different samples consisting of free, directly and indirectly coupled aP after irradiation.

Discussion

The temperature calculations have shown that under irradiation with short laser pulses gold particles can create in their surroundings temperatures which, according to the Arrhenius equation, are high enough to cause thermal denaturation of proteins. However, it is not clear whether this exponential relation between temperature and denaturation rate is also valid in the range of nano- or even picoseconds. The aim of these experiments was therefore to show that proteins in the vicinity of nanoparticles can be inactivated under laser irradiation. In our model system a significant inactivation of proteins was only observed when the proteins were bound directly to the surface of 15 nm gold particles. The control experiments without gold particles and with indirectly coupled aP showed that the laser induced damage is highly localised to the vicinity of the particles.

From the temperature calculations in Fig. 2 it can be deduced that during the irradiation a surface temperature of several thousand Kelvin was reached. This is far more than was to be expected from the Arrhenius equation for thermal denaturation of aP. Extrapolation of thermal inactivation rates of aP which were measured in a water bath with temperatures below 373 K (16) results in a temperature increase of approximately 400 K, necessary for an inactivation with 10^4 picosecond pulses (8). However, at a distance of only 5 nm from the surface the temperature had already fallen by one order of magnitude. Since the size of the aP and the antibodies is between 5 and 10 nm, the average temperature of the directly coupled proteins is considerably lower. The temperature at the indirectly coupled proteins is further reduced by at least one order of magnitude compared to the directly coupled proteins. Therefore the thermal damage mechanism is consistant with the results of these

experiments when the strong temperature gradiant is taken into account.

Beside thermal destruction, other possible damage mechanisms exist. Mechanical damage by shock waves or explosive evaporation of water (similar to laser induced cavitation) seem unlikely, since the damage range should then be larger than the 10 nm by which aP is separated from the gold particles in the aP-antibody conjugates. Photochemical destruction either by the laser induced generation of reactive molecules at the surface of the particles or by surface enhanced 2-photon absorption may cause damage which is extremely localised (8). These experiments can therefore not fully elucidate the mechanism of aP inactivation, which must be part of further investigations.

In conclusion, a highly localised laser induced protein inactivation was demonstrated. These results are very promising for high precision cell surgery with nanoparticles, which has the potential to become a new tool for modifying cells and tissues with subcellular precision.

Nanopartikelzellchirurgie hoher Präzision

Laserbestrahlung von absorbierenden Nanopartikeln mit Piko- und Nanosekundenpulsen kann prinzipiell thermische Effekte mit einer Ausdehnung unter 50 nm bewirken. Daher könnten Nanopartikel gezielt zur Erzeugung subzellulärer Effekte eingesetzt werden. Eine derartige Nanopartikelzellchirurgie könnte einzelne Proteine ausschalten, um deren Funktion zu untersuchen, oder Zellen, die sich in einem Gewebsverband befinden, manipulieren.

Konjugate aus kolloidalem Gold mit dem Enzym alkalische Phosphatase (aP) wurden als Modellsystem zur Untersuchung einer Proteininaktivierung in der Umgebung von bestrahlten Nanopartikeln eingesetzt. aP wurde entweder direkt oder indirekt über zwei Antikörper an 15 nm Goldpartikel gekoppelt. Vor und nach Bestrahlung mit Pikosekundenlaserpulsen bei einer Wellenlänge von 527 nm wurde die Proteinaktivität mittels eines Fluoreszenzverfahrens bestimmt.

Durch Bestrahlung mit 10^4 Pulsen konnte die direkt gekoppelte Phosphatase mit 50 mJ/cm^2 pro Puls fast vollständig inaktiviert werden, während bei freien nicht an das Gold gebundenen oder nur indirekt über Antikörper gekoppelten Proteinen nur ein geringer Verlust der Aktivität beobachtet wurde. Diese Ergebnisse zeigen, dass eine auf die unmittelbare Umgebung von Nanopartikeln beschränkte Inaktivierung von Proteinen durch Laserbestrahlung möglich ist. Auf den Schadensmechanismus kann aus diesen Experimenten jedoch nicht eindeutig geschlossen werden, da neben thermischer Inaktivierung auch photochemische Reaktionen die Phosphatase in der unmittelbaren Umgebung der Partikel zerstören können. Zur Aufklärung des Schädigungsmechanismus sind deshalb weitere Experimente notwendig.

Schlüsselwörter
Selektive Photothermolyse, Proteindenaturierung, Nanopartikel, kolloidales Gold

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