Inactivation of proteins by irradiation of gold nanoparticles with nano- and picosecond laser pulses

Gereon Hüttmann[*a], Benno Radt[b], Jesper Serbin[c], Reginald Birngruber[a]
[a]Medical Laser Center Lübeck, Peter-Monnik-Weg 4, D-23562 Lübeck
[b] University of Melbourne, Victoria 3010, Australia
[c]Laser Zentrum Hannover e.V., Hollerithallee 8, D-30419 Hannover

ABSTRACT

Irradiation of 15 nm gold particles with nano- and picosecond laser pulses can create locally temperatures beyond the critical point of water. Due to the short heating times the temperature is localised to the vicinity of the particles. Under irradiation with nano- and picosecond pulses an inactivation of the enzymes alkaline phosphatase and chymotrypsin which were bound to the surface of the particles was observed. As expected by strong temperature gradient caused by the short irradiation time the protein inactivation is localized within a few tens of nanometers when picosecond pulses were used. This was shown by irradiating gold protein conjugates in which the protein was bound via two anitbodies to the particle.

Keywords: nanoparticles, colloidal gold, protein inactivation, proteomics

1. INTRODUCTION

Lasers can produce extreme precise and selective effects in biological tissue[1-4]. 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 focussed to 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. High precision cell surgery by a focused beam requires high quality optics to create a submicrometer laser spot and the knowledge of the spatial localisation of the target to which the laser beam

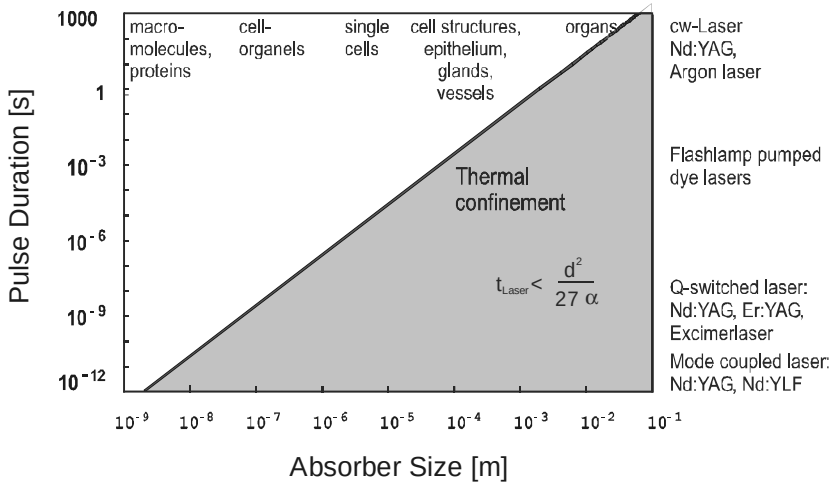

Figure 1: Pulse duration versus absorber diameter for achieving selective thermal effects.

* huettmann@mll.mu-luebeck.de; ++49-451-5006530; fax: ++49-451-505486

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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 can be brought about by photochemical reactions, as used in photodynamic therapy[5] or CALI[6, 7], 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, physical mechanisms are mostly independent of the specific target structure. Additionally, they can be influenced by the irradiation parameters. The objective of this work was to investigate whether thermal damage with subcellular precision is possible by using laser irradiated highly absorbing nanoparticles.

Prerequisite for inducing highly confined thermal damage are particles which efficiently convert the radiation to heat, and a duration of the irradiation which is short enough to prevent heat diffusion in the surroundings. Parish and Anderson proposed to use heating times shorter than the thermal relaxation time $d^2/(27\alpha)$ of the absorbing structure for a selective thermal treatment[8]. This relation, which links the laser pulse duration with the diameter d of the absorbing structure and the thermal diffusivity α of the tissue, suggests to use nano- and picosecond pulses for inducing submicrometer effects (Fig. 1). This concept of selective thermolysis was successful applied for trageting larger structures like vessels, hair follicles and pigmented cells[9-12]. It is well known, that thermal damage of proteins depends not only on the temperature but also on the duration of the heating[13,14]. Estimations on the thermal damage kinetics had shown, that even for nano- and picosecond heating times thermal damage can be expected for temperatures below 573 K[15]. By using multiple pulses it should even be possible to reduce the necessary temperature without sacrificing the

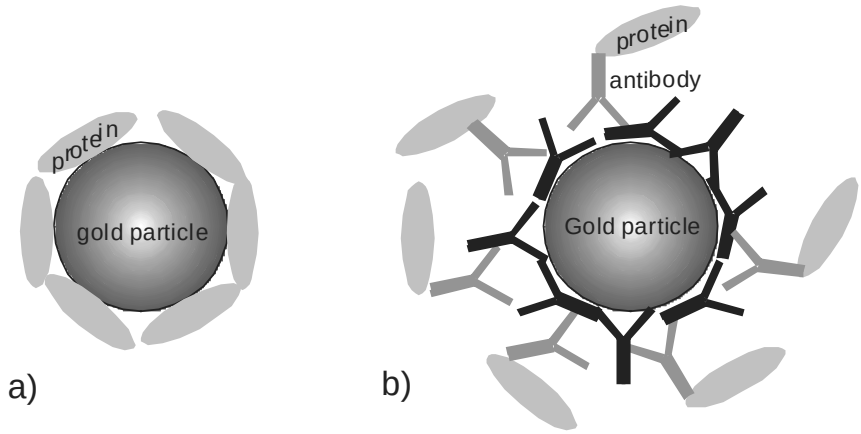


Figure 2. Protein gold conjugates used to study particle-based protein inactivation. a) Proteins coupled directly to the nanoparticle; b) proteins coupled via two antibodies

spatial resolution, since thermal effects are additive[16,17].

In order to test this concept, conjugates of the proteins alkaline phosphatase (aP-Au) and chymotrypsin (chym-Au) with gold nanoparticles were irradiated with nano- and picosecond laser pulses. The effect of the irradiation was tested by measuring the enzymatic activity of the proteins after irradiation. Gold nanoparticles were chosen as absorber, because of their high absorption cross-section[15], high temperature stability and the well established procedures for coupling with proteins.

2. MATERIAL AND METHODS

2.1 Samples

Conjugates were prepared following the usual protocols of gold antibody conjugation[18], which were adapted according to the isoelectric point of the enzymes. The exact procedure is described else were[19]. In short, the pH of a solution of 15 nm gold particles (British Biocell International) was adjusted to a pH of 5.8 by adding K_2CO_3 and HCl. The optimal concentration of alkaline phosphatase (ICN Biomedical Inc.) was determined by adding NaCl, which causes a color change by aggregation of the gold, if the particles are not fully covered with the proteins. After incubation for 10 min, unbound proteins were removed by centrifugation at 10,000 g and 2,000 g. The conjugates were stored at 4°C. Since the proteins are not covalently bound to the particles, detached proteins were removed before each experiment by centrifugation at 10,000 g. Chymotrypsin was conjugated to gold in a similar way at pH 8.8.

Conjugates in which alkaline phosphatase was not directly coupled to the gold were prepared by first coating the particles with a mouse antibody MIB1 (kindly supplied by Prof. Geerdes, FZ-Borstel, Germany) at pH 9.2. Then the solution was incubated for 30 min with an anti-mouse antibody which was covalently linked to alkaline phosphatase (DakoCytomation GmbH, Hamburg, Germany). Unbound protein was removed from the solution by centrifugation at 2,000 g.

2.2 Irradiation and enzyme assays

Samples were irradiated either by nanosecond pulses from a Q-switched frequency-doublet Nd:YAG laser (Spectron Lasers, Rugby, UK) or by picosecond pulses from a mode-locked frequency-doublet Nd:YLF laser (ISL2001 MPL, Intelligent Laser Systems Inc., USA). The lasers had a gaussian beam profile and a pulse width of 16 ns and 35 ps respectively. Irradiation with nanosecond pulses was done in sample volumes with a diameter of 0.5 mm with a gaussian beam of a similar diameter because of the limited pulse energy[20]. The radiant exposure at the rim of the volume was only 25% of the central value. One set of experiments was performed with a Nd:YAG laser with 6 ns puls duration
(YG671-10 Continuum, USA) which had a significantly higher pulse energy than the 16 ns Nd:YAG laser, so that a nearly homogeneous irradiation of the samples was possible.

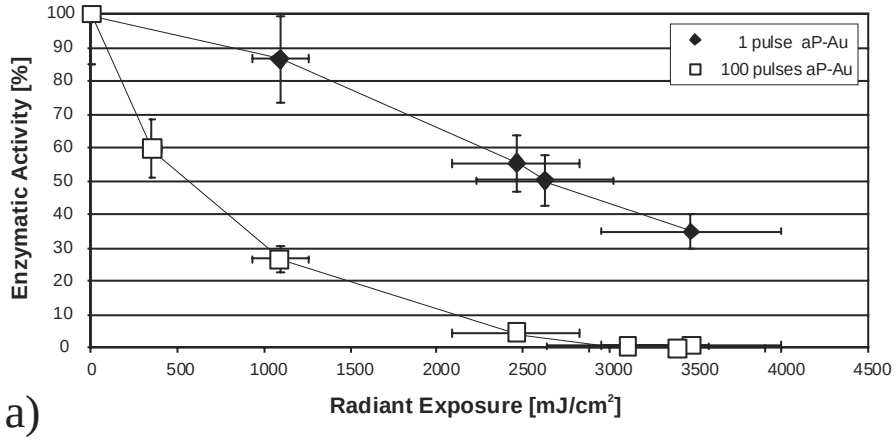
The beam diameter was measured by a pinhole, which was scanned through the beam in two orthogonal directions. After fitting a two-dimensional gaussian curve $\exp\left(-\left(\frac{x}{dx}\right)^2-\left(\frac{y}{dy}\right)^2\right)$ to the measured data the radiant exposure B in the maximum of the beam was calculated form the measured pulse energy E by $B = E / (\pi\, dx\, dy)$. All values for the radiant exposure therefore refer to the maximal value in the center of the gaussian beam.

Due to the limited pulse energy in the picosecond experiments, the beam was scanned in the sample volume, which had a diameter of 2 mm[21]. The enzymatic activity of alkaline phosphatase was measured with 4-MUP (4-Methylumbelliferylphophatase, Calbiochem), which shifts its absorption and emission band after clevage of the phophatase group. 4-MUP was used in a concentration of 10 mMol/l in DEA (Diethanolamine, Fluka) at pH 9.8. To measure the activity of chymotrypsin the substrate CII (Suc-Ala-Ala-Pro-Phe-AMC, Calbiochem) was used. Similar to 4-MUP a cleavage of the amino acids shifts absorption and emission spectra. Fluorescence measurements were done either with a commercial spectrometer (Fluoromax, Spex) or by a self build fluorescence detector. The slope of the increase of fluorescence at the choosen excitation and emission wavelength (370 nm/445 nm for alkaline phosphatase and 390 nm/450 nm for chymotrypsin) was taken as a measure for the enzymatic activity. The linear relation between slope and protein activity was checked under the experimental conditions.

Fragmentation of the gold after irradiation was studied by electron microscopy (EMP10, Phillips, Netherlands).

RESULTS

Inactivation of alkaline phosphatase and chymotrypsin was observed after irradiation with nanosecond pulses (Fig. 3). With increasing radiant exposure the activity of the proteins was reduced. With the gaussian beam profile and single pulses 50% inactivation was achieved at a radiant exposure of 2.5 J/cm^2 for alkaline phosphatase and 1.5 J/cm^2 for chymotrypsin. The slow decrease of the protein activity is mainly caused by the inhomogeneous irradiation of the samples. Therefore chymotrypsin was irradiated with a nearly homogeneous beam, which resulted in a sharp decrease of the protein activity (Fig. 3b). Additivity of the protein inactivation was also seen. Irradiation of aP-Au with 100 pulses increased the loss of protein activity.



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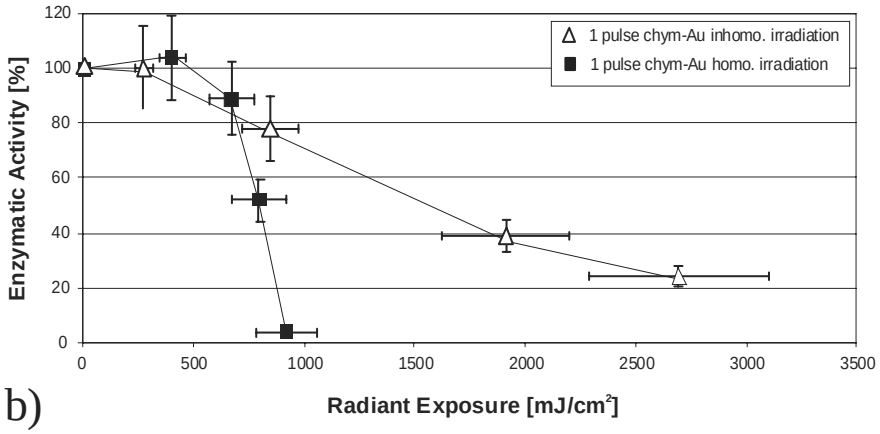


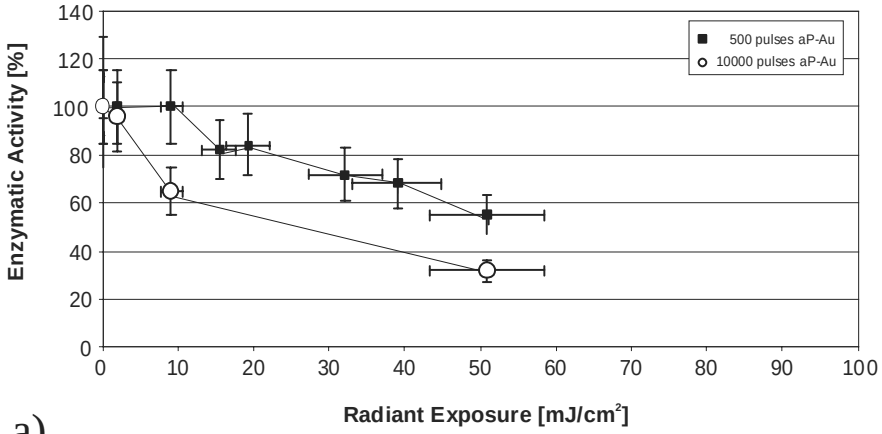
b)

Figure 3: Enzyme activity after irradiation with nanosecond pulses of different radiant exposure.
a) Irradiation of alkaline phospatase gold conjugates with one and 100 pulses.
b) Irradiation chymotrypsin gold conjugates with 16 ns gaussian and 6 ns homogenous beam.

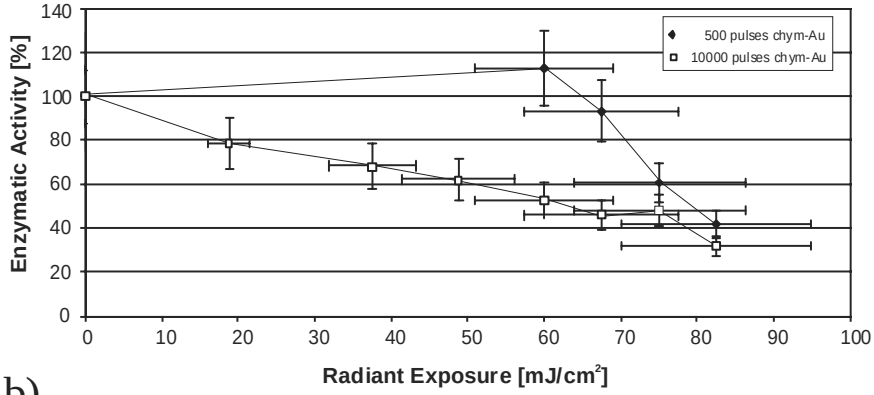
Proteins were also inactivated after irradiation with multiple picosecond pulses at a radiant exposure, which is more than an order of magnitude lower compared to the nanosecond irradiation (Fig. 4). With single pulses no inactivation was seen with an radiant exposure up to 50 mJ/cm^2. Therefore experiments were done with 500 and 10^4 pulses. Inactivation of 50% was achieved for alkaline phosphatase at lower radiant exposure (approx. 50 mJ/cm^2 with 500 pulses and 30 mJ/cm^2 with 10^4 pulses) compared to chymotrypsin (65 mJ/cm^2 for 500 pulses and 80 mJ/cm^2 for 10^4 pulses).

When conjugates with alkaline phosphatase, which was coupled via the two antibodies to the gold were irradiated with 10^4 pulses, no inactivation was observed, even at 54 mJ/cm^2, which inactivated nearly 70% of the direct bound protein (Fig. 5).

Electron microscopy of gold particles before and after irradiation revealed, that at high irradiance picosecond irradiation



a)



b)

Figure 4: Enzyme activity after irradiation with 500 and 10^4 picosecond pulses of different radiant exposure.
a) Alkaline phospatase gold conjugates. b) Chymotrypsin gold conjugates.

the particles were fragmented (Fig. 6).

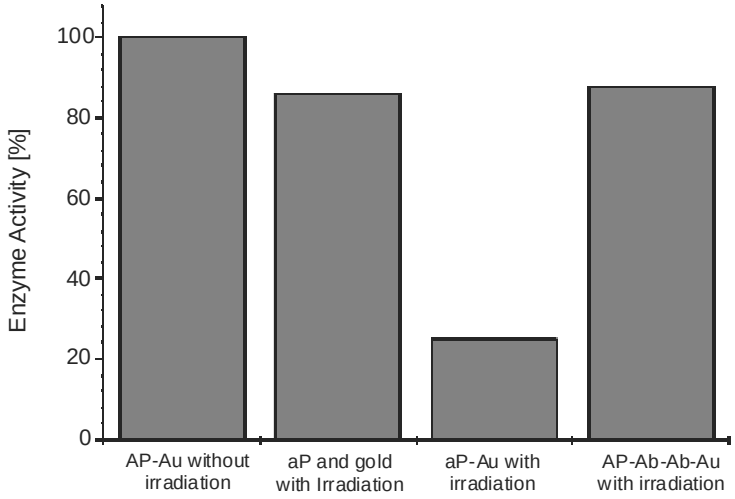


Figure 5: Inactivation experiment irradiating directly and antibody coupled alkaline phosphatase with 10^4 pulses of 54 mJ/cm^2.

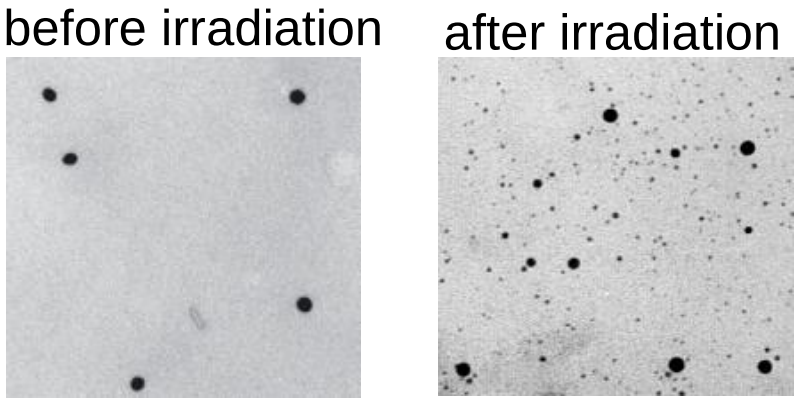


Figure 6: Electron microscopy image of alkaline phosphatase conjugates before and after irradiation with 10^4 pulses at 69 mJ/cm^2.

DISCUSSION

Gold nanoparticle have recently become very popular for the optical detection of small quantities of biological molcules like proteins or DNA[23] and as a tool for building nanostructures[24]. The unique optical properties, which manifest in the strong absorption band in the visible spectral range, make gold particle very interesting for nanoprobes. Our

experiments show that the interaction of pulsed laser light with gold nanoparticles can manipulated biological micro- and nano structures with high precision.

Since the thermal relaxation time of 15 nm gold particles is in the order of a few hundred picoseconds, heat diffusion during irradiation with picosecond pulses can be neglected, whereas during nanosecond heating the particles are nearly in thermal equilibrium with their surroundings. Therefore the temperature increase per mJ/cm^2 differs by nearly two orders of magnitude between pico- and nanosecond irradiation being 100 K and 1.2 K respectively[20, 21]. Using these numbers to estimate the surface temperatures of the particle at which the protein damage occurs, temperatures above the melting point of gold are expected in the picosecond experiments. In the nanosecond experiments the estimated temperatures are lower but are still above the critical point of water. Therefore in both cases supercritical water is expected in the vicinity of the particles, which probably leads to explosive boiling. The formation of the expanding vapor bubbles propably will change particles absorption and more important the thermal conduction from the particle in the surrounding liquid. Therefore an calculation of the actual temperatures at which the protein inactivation takes place is not possible for nanosecond pulses. Because of the strong temperature gradient and the rapid cooling these bubbles may be smaller than it was experimentally seen when irradiating micrometer-sized particles[22].

At the moment it is not clear whether the bubble formation or a different thermal or photochemical mechanism leads to the destruction of the proteins at the different pulse widths. The confinement of the damage to 10 nm when picosecond pulses are used speaks against bubble formation as a damage mechanism, since the bubbles are expected to grow larger.

These experiments showed, the laser irradiated gold nanoparticles can be used for a highly localized protein inactivation. The mechanism has yet to be identified and further experiments are under way to elucidate physical and chemical phenomena which are associated with the irradiation of gold particles. This will help to determine their potential to selectively manipulated biological structures.

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