Laser generated micro- and nanoeffects: inactivation of proteins coupled to gold nanoparticles with nano- and picosecond pulses

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ABSTRACT

Background: Protein denaturation in the fs-ns time regime is of fundamental interest for high precision applications in laser tissue interaction. Conjugates of colloidal gold coupled to proteins are presented as a model system for investigating ultrafast protein denaturation. It is expected that irradiation of such conjugates in tissue using pico- up to nanosecond laser pulses could result in effects with a spatial confinement in the regime of single macromolecules up to organelles.

Materials and Methods: Experiments were done with bovine intestinal alkaline phosphatase (aP) coupled to 15 nm colloidal Gold. This complex was irradiated at 527 nm/ 532 nm with a variable number of pico- and nanosecond pulses. The radiant exposure per pulse was varied from 2 to 50 mJ/cm^2 in the case of the picosecond pulses and 10 to 500 mJ/cm^2 in the case of the nanosecond pulses. Denaturation was detected as a loss of protein function with the help of the fluorescence substrate 4MUP.

Results and Discussion: Irradiation did result in a steady decrease of the aP activity with increasing radiant exposures and increasing number of pulses. Inactivations up to 80 % using 35 ps pulses at 527 nm with 50 mJ/cm^2 and a complete inactivation induced by 16 ns pulses at 450 mJ/cm^2 are discussed. The induced temperature in the particles and the surrounding water was calculated using Mie's formulas for the absorption of the nanometer gold particles and an analytical solution of the equations for heat diffusion. The calculated temperatures suggest that picosecond pulses heat a molecular scaled area whereas nanosecond pulses could be used for targeting larger cellular compartiments.

It is difficult to identify one of the possible damage mechanisms, i.e. thermal denaturation or formation of micro bubbles, from the dependance of the inactivation on pulse energy and number of applied pulses. Therefore experiments are needed to further elucidate the damage mechanisms. The observed inactivation dependencies on applied energy and radiant power can not be explained with one or two photon photochemistry.

In conclusion, denaturing proteins irreversibly via nanoabsorbers using pico-/ nanosecond laser pulses is possible. The expected confinement of the heat to the nanoabsorbers suggests that denaturation of proteins with nanometer precision could be possible with this approach. However, the mechanism of protein inactivation, which is part of present investigations, is crucial for the precision of such nanoeffects.

Keywords: Laser medicine, nanoparticles, colloidal gold, protein denaturation

1. INTRODUCTION

Lasers are used in a large variety of medical applications because of their ability to modify or destroy tissue with high precision. Two different ways may be used to restrict the laser-induced damage to the target volume. The laser beam can be either focused directly to the target tissue or a selective absorption in the tissue can be used to restrict damage to the target volume (Fig. 1). Using differences in tissue absorption, the temperature can be raised selectively in the stronger absorbing structures. The precision of these effects can be comparable to the size of the absorber if the damage caused by heat diffusion can be limited to the absorber's surrounding by adjusting the laser pulse width to the thermal relaxation time of the target volume. This principle of high precision laser effects, which was proposed by Parrish and Anderson,[1] is often called selective thermolysis. Several clinical applications of lasers

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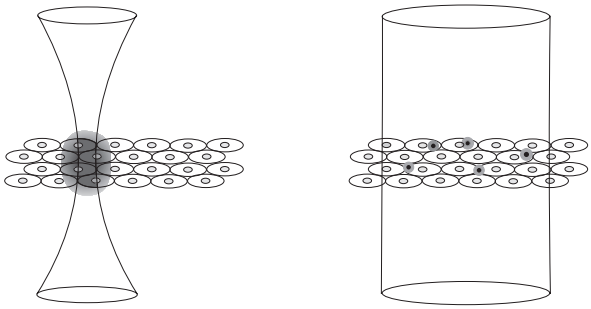


Figure 1. Localized tissue damage can be induced either by tight focusing of the laser beam (left) or by using increased absorption in the target structures (right)

rely on this principle,[2–5] which has the advantage that a scanning parallel irradiation of a large tissue area is possible without stabilizing the tissue section in relation to a focussed beam, because the targeting is only done with the help of the absorbers and not with the help of the laserbeam.

At the moment it is not clear whether also cell organels or even single macromolecules like proteins or DNA can be selectively destroyed by photothermolysis.
The main problem, when going to smaller structures, is the reduction of the laser pulse width, which is necessary to stay in the thermal confinement regime. This decreases and limits the duration of increased temperature in the tissue. Since thermal damage depends on temperature and time,[6,7] the reduction in time has to be compensated by an increased temperature. Extrapolation of rates of thermal damage of proteins and tissues towards very high temperatures shows the possibility of a selective thermal damage of biological structures near strongly absorbing nanometer-sized particles.[8] The generation of high temperatures in nanometer volumes should be possible as well, since strongly absorbing nanoparticles do not act as nucleation sides for boiling at 100 °C.[8] However, a high temperature and a very fast temperature increase can cause mechanical disruption by explosive phase transitions or shock waves, which may then destroy the precision of the laser-induced effects.

Several groups have investigated the possibility of sub-cellular thermal damage using organic chromophores..[9,10] However, if the increase of the damage temperature with decreasing heating time is taken into account, the investigated molecules will rather undergo photochemistry than cause thermal damage as calculations have shown.[8,11]

The aim of this work was to investigate the possibility of protein inactivation on an ultrashort time scale by the irradiation of protein-gold conjugates. Conjugates made of colloidal gold with a diameter of 15 nm with alkaline phosphatase (aP) were chosen for the experiments, because alkaline phosphatase is well known in structure, biochemical behaviour and is commonly used for a variety of enzymatic assays. These conjugates were irradiated with nanosecond laser pulses. After irradiation the residual activity of the proteins was measured by a fluorescence assay. The results are compared to results of picosecond irradaition experiments[12]in order to validate that denaturation of proteins is possible with different laserpulse and particle size parameters that should result in a molecular up to macromolecular spatial resolution of the effect. The aim in the nanosecond experiments was to further elucidate the denaturing effect and to prove that simple Q- switch pulses could be used for targeting subcellular structures like protein clusters or cell organells via gold conjugates.

2. MATERIALS AND METHODS

2.1. Samples and measurement of the protein activity

The conjugates of 15 nm gold particles with bovine intestinal alkaline phosphatase were produced following usual protocols of antibody gold conjugation[13] which were adapted to conjugate the enzymes at the proper isoelectric point of pH 5.8. 50 mM Phosphatebuffer was used as conjugation buffer and was adjusted to pH 5.8 using the colloidal gold stock solution at pH 5. The colloidal gold was purchased from British Biocell International.The enzymes used (sigma p5521)were dialyzed before use for 24 h at 4°C. Although the conjugates can be stored at 4 °C for several weeks, a certain amount of protein separates from the gold particles during storage because the proteins are not covalently

bound to the particle surface. Therefore, in the beginning of each experiment unbound proteins were removed by centrifugation at 15000 g for 45 min. The loose pellet was carefully transferred with a pipette to a new vial and was resuspended in 50 mM phosphate buffer (pH 7.6). After this procedure, the aP activity of unbound protein was less than 25% of the activity of the aP-Gold conjugates. Additionally, before and after irradiation the activity of the bound and free aP was measured.

The activity of alkaline Phosphatase was measured with the fluorescent substrate 4-MUP (4-Methylumbelliferyl-phosphate, Calbiochem) with Diethanolamine buffer added, resulting in an overall pH 9.8. The aP activity was measured with a self built fluorescence detector which was equipped with an excitation bandpass at 370 nm and an emission bandpass at 445 nm. A conversion of 4-MUP to 4-MU through the catalytic action of aP results in an increase of the measured fluorescence signal with time. Using different concentrations of aP, it was checked that under our experimental conditions the slope of the fluorescence increase was in a linear relationship to the concentration of active aP.

2.2. Irradiation of the samples

The irradiation of the samples in the picosecond experiments was done with 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. The laser gives a stable output energy with 5% standard deviation and a Gaussian intensity distribution.[14] The irradiation of the samples in the nanosecond experiments was done with a Q-switched frequency doubled Nd:YAG. The maximum pulse energy at 532 nm was 5 mJ at TEM00. The pulse duration was 16 ns FWHM.

The sample diameter was 2 mm (volume of 3.5 μl) in the picosecond experiments.[12] In the nanosecond experiments, the diameter was chosen to be 500 μm (volume 1.6 nl). The sample size was reduced due to the limited laser energy with the aim to achieve a radiant exposure as homogenous as possible by just using the tip of the gaussian beam profile. The sample glass slides were positioned under a microscope, at which the illumination system was replaced by a dichroic mirror as described in.[12] For the irradiation the laser beam diameter was adjusted to the sample size and the desired irradiation with the help of a telescope.
In the first set of experiments using an expanded beam radiant exposures were measured by averaging the energy over the irradiated area. The maximum radiant exposure was 2 mJ/cm^2 in the picosecond experiments and 100 mJ/cm^2 in the nanosecond experiments respectively at maximum pulse energy. The irradiance at the rim of the samples irradiated with the picosecond pulses was 70% of the maximum irradiance in the center (Fig. 2a and c). The irradiance in the nanosecond experiments at the rim of the samples was 90 % in relation to the center irradiance.
In order to achieve higher radiant exposures more inhomogeneities within the sample were allowed. The higher radiant exposures were realized following two strategies:
In the picosecond experiments, the unexpanded beam, which had a diameter of 0.5 mm, was directed to a field of 4x4 spots in the sample, which had a distance of 0.5 mm from each other (Fig. 2b). With this approach a nearly homogeneous total radiant exposure of up to 50 mJ/cm^2 per pulse was achieved (Fig. 2d).

In the nanosecond experiments higher radiant exposures were realized by compressing the 6 mm laserbeam with a telescope to the sample size, which was chosen 4 times smaller in comparison to the picosecond experiments for reaching higher radiant exposures with a tolerable inhomogeneity. The irradiance at the rim of the sample was 30 % of the center irradiance hence the ihomogeniaties within the sample were strong in spite of the attempt to allow a homogenous irradiation.

Laser	sample dia.	volume	maximum radiant exposure
Nd:YLF; 35 ps	2 mm	3.5 μl	16 fields scanned (50 mJ/cm^2)
Nd: YAG 16 ns	500 μm	1.6 nl	focussed beam (450 mJ/cm^2)

2.3. Temperature calculations

The temperature at the surface and in the surrounding of gold particles in water was calculated using Mie's formulars for the efficiency factor of absorption Q_{abs} and an analytical solution of the differential equations for heat diffusion as described in.[12]

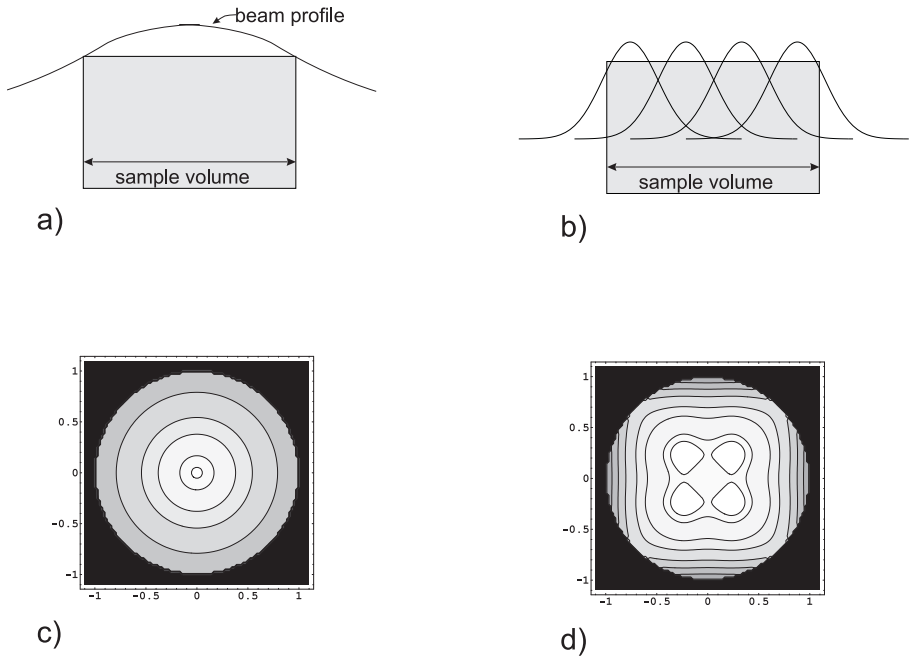


Figure 2. Distribution of the radiant exposure inside the sample volume. a), c) Irradiation with the expanded beam. b), d) Scanned irradiation with a 0.5 mm beam. c) and d) show a contour plot of the total radiant exposure. The contour lines were drawn at 50%, 60%, 70%, 80%, 90%, 95%, 99%, and 99.9% of the maximum.

3. RESULTS

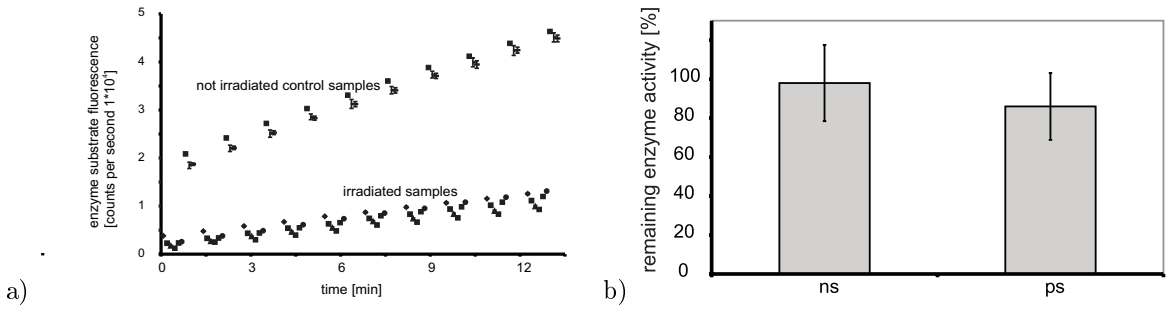
3.1. Irradiation of the conjugates

The conjugate activity was observable as a fluorescence increase in the samples. Results shown in Figure (Fig. 3) are examples for fluorescence signals which clearly show a lower fluorescence increase in 6 irradiated samples (100 pulses @ 400 mJ/cm^2 16 ns)compared to three not irradiated controls. The ascending signal slope was calculated from the fluorescence intensity increase of each sample, which is proportional to the activity of the aP in the samples.

The result of all experiments with radiant exposures of max. 2 mJ/cm^2 and 100 mJ/cm^2 in nanosecond experiments was that alkaline Phosphatase was not denatured irreversibly by the irradiation. No inactivation of the irradiated aP-gold conjugates was observed, neither after an application of 10^4 picosecond pulses nor an irradiation using 100 nanosecond pulses.

Experiments using increased radiant exposures lead to an inactivation of the conjugated proteins. Irradiation with nanosecond pulses and high radiant exposures which were realized by reducing the laser beam and sample diameter as described in the previous section reduced the alkaline Phosphatase activity in the samples down to no residual activity (Fig. 3). In the case of the picosecond experiments, a higher radiant exposure was realized by scanning the sample using 4 times 4 beam positions in an overlapping way. Inactivations of up to 80 % were obtained using multiple pulses. As a control experiment, solution of unbound alkaline Phosphatase was irradiated under the same conditions at maximum radiant exposures. Only a slight reduction of the aP activity was observed (Fig. 3) after ps irradiation. In nanosecond control experiments no inactivation of unconjugated alkaline Phosphatase was observed.

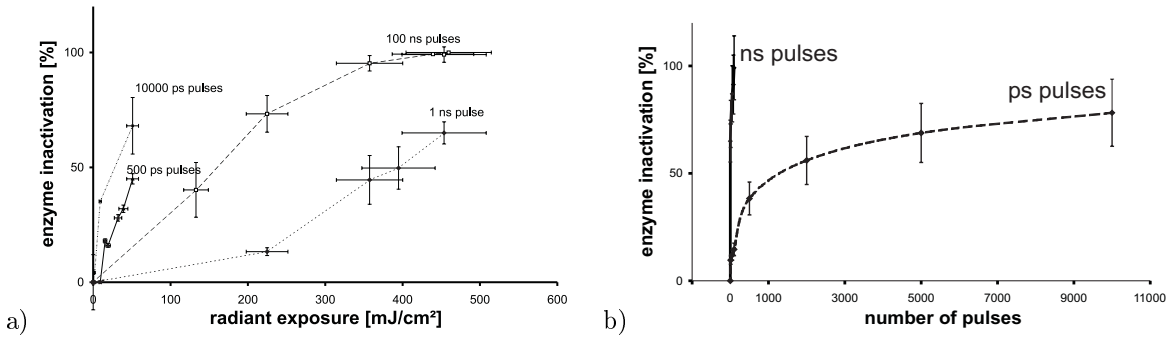
The inactivation of aP (IA) was defined by the loss of activity due to the irradiation in relation to the protein activity without irradiation. Even though unbound aP was removed from the samples before irradiation by centrifugation, there was always a certain amount of unbound protein in the sample, which cannot be inactivated by the irradiation. In order to correct the measured inactivation for this error, a fraction of the sample solution was spun down before the experiments were conducted, and the activity of the sediment A_{Sed} and the supernatant A_{Sup} were



a) b)

Figure 3. Laser induced Inactivation of aP bound to 15 nm gold particles in comparison to no inactivation of unconjugated enzymes
a) Activity of alkaline Phosphatase after irradiation with 100 16 ns pulse at 532 nm. Slope of the fluorescence of 6 samples irradiated with 100 pulses 300 mJ/cm^2 and three control samples. Significant inactivation is observable in the reduced fluorescence increase of the irradiated samples which is proportional to the residual enzyme activity.
b)Activity of free alkaline Phosphatase in solution, after irradiation with maximum radiant exposure applied in all experiments(ps: 10000 pulses; 50 mJ/cm^2 - ns: 100 pulses 450 mJ/cm^2).

measured. The inactivation IA was corrected by the factor $(A_{Sed} - A_{Sup})/A_{Sed}$. Protein activity was measured for different radiant exposures and different numbers of pulses and the corrected inactivation was calculated from these measurements (Fig. 3).



a) b)

Figure 4. Inactivation of aP bound to 15 nm gold particles.
a) Dependence of the inactivation on the radiant exposure for 500 and 10^4 picosecond pulses (35 ps) and for 1 and 100 nanosecond pulses (16 ns).
b) Dependence of the inactivation on the number of pulses at maximum radiant exposures of 50 mJ/cm^2 in the picosecond experiments and 450 mJ/cm^2 in the nanosecond experiments.

With single nanosecond pulses, an inactivation was observed above radiant exposures of 200 mJ/cm^2 . The inactivation increases with increasing radiant exposure more ore less linearly. Using multiple pulses, again there is an increasing inactivation with increasing radiant exposure that flattens towards 100% inactivation. Using 100 pulses a complete inactivation can be achieved at 450 mJ/cm^2 . The results of the picosecond experiments show a homologous dependency of increasing inactivation with increasing radiant exposure. There is one important difference: The applied radiant exposures which are proportional to the energies deposited within the heated volume are lower by a factor of 10. With 500 pulses, the protein activity was reduced with a radiant exposure above 10 mJ/cm^2 (Fig. 4a). The inactivation increases linearly up to 60% for radiant exposures increasing up to 50 mJ/cm^2 . After an irradiation with 10^4 pulses the effect was even stronger. 10 mJ/cm^2 gave nearly 40 % inactivation and with 50 mJ/cm^2 the activity was reduced by almost 70 %. No complete inactivation was induced with the applied

maximum radiant exposure which was limited by the used laser system.

The dependence of the inactivation on the number of pulses is shown in Figure (Fig. 4b). The inactivations were measured for 50 mJ/cm^2 using picosecond pulses and 450 mJ/cm^2 using nanosecond pulses. The aP inactivation increases nonlinearly with increasing number of pulses. The inactivation rises sharply in the case of the nanosecond experiments, whereas in the case of the picosecond experiments there is only a weak inactivation increase with the applied number of pulses.

In summary, an irreversible inactivation of the proteins is possible using ns and ps irradiation parameters. There is a certain additivity of the effect and the inactivation energies deposited within the conjugate surroundings are higher by a factor of ten in the nanosecond experiments compared to the picosecond experiments.

3.2. Temperature calculation

The temperature distribution inside and in the surrounding of a 15 nm gold particle which is immersed in water was calculated for the applied laser pulses. Since the thermal diffusivity of gold is nearly 900 times larger than that of water, the temperature inside the particle stays constant across the radius during the cooling: The temperature in the center exceeds the temperature at the rim only by a few percent. In contrast, in the surrounding of the particle the temperature decreases quite rapidly. Using picosecond pulses temperatures at a distance of 10 nm apart from the particle surface reach less than 5% of the surface temperature. 10 nm is the approximate size of aP, which is bound to the surface of the gold particles. Therefore, a strong temperature gradient along the proteins is expected. After the end of the laser pulse, the particle rapidly cools down within picoseconds (Fig. 5b). In contrast to the very steep temperature gradients that can be expected in the case of the picosecond irradiation the application of nanosecond pulses leads to a 10 ns temperature jump and a larger heated volume and lower peak temperaures due to heat conduction. The larger heated volume becomes obvious in the plotted temperature curves for the temperatures in 5 nm and 10 nm distance from the absorber surface. The relative temperature increase in 10 nm distance is 0.3 in the nanosecond experiments whereas the relative temperature is lower by a factor of 20 if the particles are heated with picosecond pulses. The induced maximum temperature is 100 K/ mJ/cm^2 using picosecond pulses and still 1.6 K/ mJ/cm^2 during nanosecond irradiation.

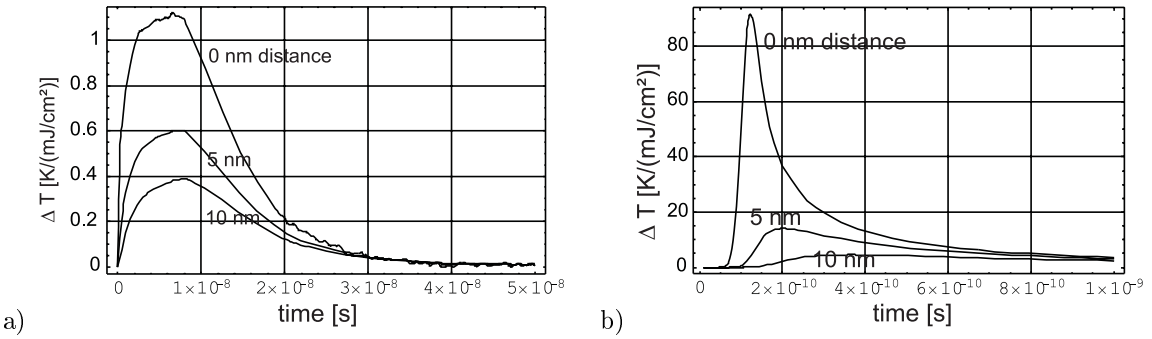


Figure 5. a) Change of the temperature with time of a 15 nm gold particle irradiated with 16 ns Q-switch pulses at the particle surface, in 5 nm and 10 nm distance from the surface. b) Change of the temperature with time of a 15 nm gold particle irradiated with 35 ps pulses at the surface, in 5 nm and 10 nm distance from the surface.

4. DISCUSSION

Aim of the experiments was to investigate whether it is possible to inactivate proteins, which are bound to nanometer-sized gold particles, by short laser pulses. The irreversible denaturation of proteins on the nanosecond time scale is supposed to be a prerequisite for using such heated particles in order to inactivate molecules or organelles in or on cells. Short laserpulses were used in order to prevent heat diffusion to the surrounding with the application in mind that cellular proteins could be inactivated without heating the entire cell. Due to the high absorption of the gold particles, extremely high temperatures can be reached at the surface of the particles. For example in the case of 35 ps pulses, with every mJ radiant exposure the temperature is increased by approximately 100 K. These high

temperatures are localized in a volume with a diameter of 35 nm and last only a few hundred picoseconds. Under such conditions, a reduction of the activity of proteins which are bound to the gold particles was observed. As multiple pulses were used, the degree of inactivation increased with the number of pulses. Therefore, a certain additivity of the effect was observed. Assuming that all pulses act independently from each other, an exponential dependence of IA on the number of pulses is expected:

$$IA(N) = 1 - \mathrm{e}^{-\alpha N} \tag{1}$$

α gives the probability that a protein is damaged by one pulse. The measured increase of the inactivation with the number of pulses used for irradiation shows roughly this relationship in nanosecond as well as in picosecond experiments. Making use of pulse numbers higher than the applied 10000 pulses in the ps experiments is not convincing because the inactivation increase per pulse decreases exponentially and is very low towards high pulse numbers.

The dependence of the inactivation on the radiant exposure is indicative for the damage mechanism. The observed nearly linear decrease in aP activity with radiant exposure goes down to 40% residual activity in the case of the picosecond experiments and in the case of the nanosecond experiments even no residual activity is left after an application of the maximum radiant exposures. The observed continuous inactivation increase ending in a residual activity in all experiamental series beside the 100 pulse/ 16ns experiments indicates that an inactivation of the conjugates is possible for nanosecond and picosecond irradiations at higher radiant exposures.

Possible damage mechanisms are a thermal inactivation, mechanical destruction due to shock waves or evaporation of the water and photochemistry. In principle a thermal inactivation of aP could be possible within 10 ps if typical protein denaturation rates are extrapolated towards high temperatures.[8] From inactivation experiments of aP solutions which were heated in a water bath, a frequency factor $A_0 = 1.85 \times 10^{23}$ and activation energy $E_a =$ 161 kJ/mol were obtained as parameters for the Arrhenius equation, which describes the thermal damage rate of aP k_{TD} at temperatures below 100°C.[14]

$$k_{TD} = A_0 \mathrm{e}^{-\frac{E_a}{RT}} \tag{2}$$

R is the universal gas constant. If the Arrhenius equation is extrapolated to damage rates of 10^{11} 1/s, a temperature of 450°C is expected to be necessary for a thermal protein inactivation within 10 ps, respectively, a temperature of 280°C for 10 ns temperature jumps is expected. In general a sharp increase of the inactivation with increasing radiant exposure is expected for a thermal damage mechanism, since the damage rates depend exponentially on the temperature. This was not observed in the experiments, which may be due to two different effects. According to the temperature calculations (Fig. 5), these temperatures are achieved at the surface with radiant powers of 4.5 mJ/cm^{2}ps; 173 mJ/cm^{2}10ns, whereas at a distance of 10 nm at least 90 mJ/cm^{2}35ps; 433 mJ/cm^{2}10ns are necessary to reach sufficiently high denaturation rates. Thus even if the extrapolation of the the Arrhenius equation into the nanosecond and picosecond range gave valid data, the extreme temperature gradient across the 10 nm extended proteins makes it very difficult to compare the results of the experiment with the Arrhenius theory and flattens the inactivation curves. Secondly, the sharp increase of the inactivation is washed out because the damage rates varie strongly within the sample, being highest at maxima of the gaussian beam positions.[14]

As shown in the temperature calculations, temperatures well high above 100 °C and even above the critcal point of water can be expected. This may be realistic due to the very small heated volume without classical nucleation sides inside and the very short heating period. Nevertheless, as soon as water is evaporated a nanometer scaled gas bubble may be formed which insulates the particle from the surrounding water. In this case the temperature could rise significantly, since in the absence of heat conduction. 1 mJ/cm^2 causes a temperature increase of 285 K instead of 100 K in this case. Such a superheated vapor bubble leading to a explosive phase transition may also destroy the proteins on the absorbers.

Photochemical processes have to be discussed as a third damage mechanism. Classical photochemistry can be excluded as a damage mechanism, because the damage depends not only on the applied energy. This becomes obvious if the results obtained with differend numbers of pulses and radiant exposures are compared. Still a direct 2-photon excitation of the proteins may be resposible for the inactivation. Aromatic amino acids decompose with a quantum efficiency η_{PD} of a few percent if excited with cw-irradiation at 254 nm.[15] For an irradiation with N the damage rate k_{PD} can be estimated with following equation:

$$k_{PD} = N\ \eta_{PD}\ \sigma_{2ph}\ \left(\frac{E}{\tau \frac{h\ c_0}{\lambda}}\right)^2 \tag{3}$$

Assuming an 2-photon absorption cross-section σ_{2ph} of 10^{-50} cm^4s/photons,[16] and a quantum efficiency for photodamage of 5% the calculated damage rates caused by the 2-photon absorption at the energies corresponding to inactivations of 50 % are:

Laser	radiant exposure causing 50% denaturation	Number of pulses	2 photon damage rates
Nd:Ylf ;35 ps	$55\,mJ/cm^2$	500	$4.36 * 10^6$
Nd:Ylf ;35 ps	$27.5\,mJ/cm^2$	10000	$2.17 * 10^7$
Nd:Yag ;10 ns	$395\,mJ/cm^2$	1	6
Nd:Yag ;10 ns	$160\,mJ/cm^2$	100	91

The rates are approximately three orders of magnitude too slow in the picosecond experiments in order to destroy the proteins during the pulse width τ, which was 35 ps. A destruction of free proteins in solution is therefore not expected with these laser parameters. In the case of nanosecond pulses, damage rates are that slow that, even if surface effects which can increase the electromagnetic fields by several orders of magnitude are taken into account it is unlikely that proteins are destroyed by "a surface enhanced" 2-photon photochemistry. two different mechanisms are possibly responisible for the inactivation. Under irradiation with the picosecond pulses, electrons may be ejected from the gold particles into water,[17] which oxidize the amino acids in the aP.

In conclusion, protein inactivation is possible via nanoparticles and short laser pulses. The expected confinement of the heat to the nanoabsorbers when irradiating with pulses fitting the thermal realxation time of target volume suggests that a denaturation of single proteins in cells could be possible with this approach using ps pulses. By applying longer pulses like nanosecond pulses the damage radius can be increased theoretically. However, the mechanism of protein inactivation is still part of the investigations and only direct photochemistry is unlikely to be responsible for the inactivation mechanism, which is crucial for the precision of these nanoeffects.

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