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# In situ bioconjugation—Novel laser based approach to pure nanoparticle-conjugates

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ABSTRACT

The generation and characterization of nanoparticulate carrier systems is important for drug delivery, biosensing and in vivo or in vitro diagnostics. Conventional nanoparticle generation is based on chemical synthesis methods requiring time intensive reaction and additive design for each material. Successive purification and surface functionalisation is often required after the nanoparticle generation to achieve pure nanoparticle-bioconjugates. We established a novel single step method, which allows the generation of pure nanoparticles and their in situ conjugation with biomolecules bearing electron donating moieties using pulsed laser ablation in liquids. For comparison between unspecific binding and binding through strong dative bonds (here: S-Au), we applied this preparation method to the conjugation of gold nanoparticles with unmodified and thiolated oligonucleotides. In order to determine optimal parameters (laser pulse energy, focus diameter), the influence on productivity of nanoparticle generation and their interaction with oligonucleotides is studied. We report quenching of nanoparticle growth and modification of the surface plasmon resonance as evidence of a successful functionalisation. Their stability in ionic solutions is evidenced with relevance to biological and medical assays. Negligible differences between the two model bioconjugations evidence the universality of the established in situ bioconjugation method.



## 1. Introduction

The generation and characterization of nanoparticulate carrier systems is important for drug delivery, biosensing and in vivo or in vitro diagnostics. Those systems seem to penetrate into cells and to induce a certain function depending on nature and surface activity (e.g. hyperthermia [1], imaging [2], gene regulation [3] or antisensing). Especially, gold nanoparticles are of increasing interest for cell imaging [4] and sensing due to their inert properties concerning cytotoxicity and their higher magnitude of light scattering compared to molecular fluorophores [5].

Any process designed to generate such nanoparticulate systems needs to consider the application prospects of generated nanoparticles and the time required for generation. Conventional nanoparticle generation is well established by chemical synthesis methods. The required functions for targeting, enhanced cellular uptake, etc. can be achieved in a successive surface functionalisation after the nanoparticle generation. However, this requires time intensive adaptation for each material and their biomedical application might be restricted due to the risk of impurities, caused by the use of additives and chemical precursors [6].

The present paper introduces a novel preparation method, enabling a single step generation of pure nanoparticle-conjugates by laser ablation in liquids. This method consists in the laser ablation of a target in liquid media containing conjunctive agents. The approach takes advantage of the purity of nanoparticles, generated by laser ablation of solid targets in liquids without the use of chemical precursors [7–12]. Laser generated gold nanoparticles show electron accepting properties due to a partial oxidation. This property is a prerequisite for in situ functionalisation, as it implies a tendency of dispersed additives bearing electron donor moieties as $NH_2$, COOH, SH, S–S, etc. to coordinate to the nanoparticle's surface.

Oligonucleotides were chosen as model substances for bioconjugation, so that we added single stranded oligonucleotides (ssO) to deionised water prior to ablating a gold foil. The novel approach is discussed with regard to optimal laser and process parameters, which are evaluated respective the integrity of biomolecules and the productivity of nanoparticle generation. For comparison between unspecific binding and binding through strong dative bonds (here: S-Au), we performed experiments with

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unmodified (ssO) and thiolated ssO (ssOSH). The size and morphology of the resulting conjugates (ssO-Au, ssOSH-Au) is characterized by transmission electron microscopy (TEM) and results are compared to non-conjugated gold nanoparticles, generated under equal conditions. With regard to biological and medical testing, the stability in high ionic strength solution is determined.

## 2. Experimentals

### 2.1. Chemicals

The oligonucleotides were purchased from Biospring GmbH (D-60386 Frankfurt am Main) (ssOSH: 5′-cta-cct-gca-ctg-taa-gca-ctt-tg-3′, Mod. 5′:C6-Disulfide, ssO without modification) and the gold foil (thickness: 0.1 μm, purity > 99.99%) from Goodfellow GmbH (D-61213 Bad Nauheim). The ssOSH omitted the treatment with dithiothreitol (DTT) prior to conjugation, which is usually carried out to ensure that the thiol modified oligonucleotide is in a mono-thiol form rather than disulfide when applied to nanoparticles. Dougan et al. observed that the conjugation process is even improved by using alkyl-thiolated oligonucleotides since there is no trade off in conjugate stability or surface coverage as a result of omitting reduction with DTT [13].

### 2.2. Generation method

Laser generation of gold nanoparticles was carried out using a femtosecond laser system (Spitfire Pro, Spectra-Physics) delivering 120 fs laser pulses at a wavelength of 800 nm at a repetition rate of 5 kHz (maximum energy: 400 μJ per pulse, beam diameter: 4 mm). A focal length of 40 mm was applied. Unless otherwise indicated, 100 μJ pulse energy and a focal position of −2 mm (spot diameter: 270 μm), relative to the focus in air, were chosen due to the degradation and stabilization yield determinations. The principle set-up is described as follows: a 5 mm × 5 mm gold foil, thoroughly cleaned, was placed on the bottom of a petri dish filled with 500 μL aqueous solution of ssO/ssOSH (3 μM, $H_2O$: >18 MΩ cm). The depth of the layer above the target was 4 mm. The plate was placed on an axis-system, that moved at constant speed of 1 mm $s^{-1}$ in a spiral with outer radius of 1 mm and inner radius of 0.4 mm. Time of irradiation was fixed to 53 s.

### 2.3. Characterization methods

UV/vis spectra of the colloidal solutions were recorded in the spectral region 230–800 nm, with a Shimadzu 1650.

A transmission electron microscope (TEM Philips CM30) with 0.23 nm point to point resolution was used to obtain micrographs of the colloids. Samples were prepared by placing a drop of the solution on a carbon coated, formvar-covered copper grid, and dried at room temperature. The diameters of 700 particles were measured to obtain a particle size distribution.

ζ-Potential measurements were performed with the Zetasizer ZS (Malvern). In general 200 μL of the colloid were added to 800 μL water and the resulting dilution was injected into the electrophoretic cell. Three consecutive measurements were carried out.

For the determination of the stabilization yield, the gold foils were weighted three times prior and after ablation on a Sartorius balance (accuracy: 1 μg).

For the investigation of resulting traces on the gold target, 5000 pulses were released at a pulse energy of 200 μJ varying the focal position (from +3 to −8 mm, relative to the focus in air defined at 0 mm). The resulting spot diameters were analysed afterwards via light microscopy (Olympus a4i DIG 3300).

### 2.4. Degradation studies

ssOSH-Au were generated according to the generation method. Various pulse energies (from 50 to 200 μJ) and focal positions (from 0 to −8 mm) were applied. The media was then analysed by gel electrophoresis on a 20% polyacrylamid gel. After nanoparticle generation the ablation media were treated with DTT (100 mM). DTT was used to rapidly displace the surface bound ssOSH and induce nanoparticle aggregation as consequence. This method guarantees that both, conjugated and unconjugated ssOSH are analysed by gel electrophoresis. Before denaturing and mixing with loading buffer, the samples were centrifuged to ensure that all nanoparticulate sediment was separated from the supernatant. Gels were submerged in 1× TBE and run for 3 h at 300 V. The references (containing the same concentration of ssOSH) omitted the laser irradiation and were treated equally before gel electrophoresis and run next to the samples on the same gel. To visualize the bands on the gel after electrophoresis, the gels were stained and developed using Fast*Silver*™ (G Biosciences, USA). Scans of resulting stained gels were analysed with Image J. The intensities of the bands ($I(x)$) from the samples were compared to the bands of the references ($I(0)$) in order to deduce the degree of degraded biomolecule by the following equation:

$$\mathrm{Deg}(x) = 1 - \frac{I(x)}{I(0)} \quad (1)$$

## 3. Results

### 3.1. Influence of laser and process parameters

Generally, the ablation efficiency and nanoparticle yield increases with increasing laser fluence, meaning with high pulse energies and small spot sizes at focal positions close to zero. But at the same time, the heat impact to the material and the risk of heating-induced degradation increases as well. Optimal laser and process parameters used for bioconjugating nanoparticles in situ have to achieve a negligible degradation of biomolecules after interaction with the laser beam and a sufficient productivity of nanoparticle generation.

In order to determine a process window meeting these conditions, we first studied the degree of ssOSH-degradation at various pulse energies and focal positions by gel electrophoresis (Fig. 1a). The amount of intact ssO decreases with increasing applied pulse energy. Regarding the focal position, one observes a maximal degradation at −2 mm. To guarantee the integrity of ssO, the in situ conjugation process can be carried out in a broad parameter window, located in the light green field of Fig. 1a. Within this window, we determined the productivity of nanoparticle generation at same experimental conditions (Fig. 1b). We observed a reverse profile compared to the integrity of ssOSH. Maximal nanoparticle productivity up to 50 μg/min is achieved at a focal position of −2 mm. Further in situ bioconjugations are carried out at 100 μJ, −2 mm for 53 s, since these parameters seem to display a compromise between nanoparticle productivity and integrity of ssO. Under these conditions, the nanoparticle-bioconjugate productivity is 20 μg/min and the integrity of the oligonucleotide is 100%.

### 3.2. Bioconjugate-characterization

Using these optimal parameters, we generated bioconjugates and pure gold nanoparticles for characterization of optical property,