# A Self-Reporting Tetrazole Based Linker for the Biofunctionalization of Gold Nanorods

Lukas Stolzer,[a,b] Antonina Vigovskaya,[a] Christopher Barner-Kowollik*[b,c] and Ljiljana Fruk*[a]

**Abstract:** A photochemical approach based on nitrile imine-mediated tetrazole-ene cycloaddition is introduced to functionalize gold nanorods with biomolecules. For this purpose, a bifunctional, photoreactive linker containing thioctic acid as the Au anchoring group and a tetrazole moiety for the light-induced reaction with maleimide-capped DNA was prepared. The tetrazole based reaction on the nanoparticles' surface results in a fluorescent pyrazoline product allowing for the spectroscopic monitoring of the reaction. This first example of NITEC-mediated biofunctionalization of Au nanorods paves the way to the attachment of other sensitive biomolecules, such as antibodies and other proteins, under mild conditions and expands the toolbox for the tailoring of nanomaterials.

During the last decades very significant progress has been made in the synthesis, functionalization and application of engineered nanomaterials such as nanoparticles (NP) in the biomedical fields such as biosensing, bioimaging or drug delivery.[1] For such applications it is particularly important to prepare biocompatible, stable and water soluble nanomaterials, which can additionally be functionalized with various stabilizing or targeting molecules such as polymers and/or biomolecules (peptides, antibodies, nucleic acids). Various mild, high yielding and, since recently, orthogonal ligation strategies have been employed to enable the attachment of functional molecules to the NP surface.[2] Among them, modular ligation strategies often fulfilling the click chemistry criteria[3] overcome some of the issues such as low selectivity and low yields encountered with standard ligation approaches, i.e. the commonly used amide coupling.[4] Click chemistry based strategies such as the copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition (CuAAC) have already succesfully been used for the biofunctionalization of NPs.[5] For instance, Murphy *et al.* employed a two step procedure to introduce azide groups to the surface of gold nanorods (Au NR) by amide coupling and performed a CuAAC reaction to immobilize an acetylene-modified enzyme trypsin.[6] There are also reports of the attachment of fluorophores,[7] peptides[8] and a range of functional proteins.[9] However, the use of cytotoxic copper catalysts,[10] traces of which could be transferred to subsequent reactions due to the difficult work up, has disadvantages, in particular when NPs are used for *in vivo* studies. Thus, efforts have been made to utilize copper-free dipolar cycloadditions employing strained cyclooctynes and azides, which can be also used for coupling within cells.[11] Recently, such strain-promoted alkyne-azide cycloadditions were employed to decorate azide-functionalized Au NPs with cyclooctyne-modified arginylglycylaspartic acid (RGD) peptides to target integrin $\alpha_v\beta_3$ receptors and act as tumor or angiogenesis marker.[12] In addition to alkyne-azide based Huisgen cycloadditions, mild biofunctionalisation of NPs in aqueous environment was also achieved by Diels-Alder reactions. For example, Chen *et al.* conjugated furan-modified DNA to maleimide-coated silver NPs through Diels-Alder cycloaddition in presence of LiCl.[13] However, despite the progress in the last years, there is still a great demand for an universal and versatile approach to functionalize metallic NPs with biomolecules.[14] Particularly interesting are reactions that employ light as trigger as a high level of spatial and temporal control over the reactions can be achieved.[15] Such processes have already been used to induce molecular self-assembly, to exert control over photosensitive molecules,[16] modify behaviour of the labelled biomolecules and induce reactive oxygen species production by nanoparticles.[17] However, despite the significant potential, there exist only limited examples for light-induced reactions on NP surfaces. For example, Smith *et al.* demonstrated the photochemically triggered assembly of Au NPs and $ZrO_2$ NPs through [4+4] cycloaddition of adsorbed anthracene.[18] We have, on the other side, recently shown that phototriggered Diels-Alder reactions can be employed to modify silver NPs with antifouling and biocompatible polymers[19] or pattern Au NPs onto surfaces using direct laser writing.[20] Herein, we, describe the use of the nitrile imine-mediated tetrazole-ene cycloaddition (NITEC) for the biofunctionalization of Au NRs (Fig. 1), which, due to their strong, IR-induced

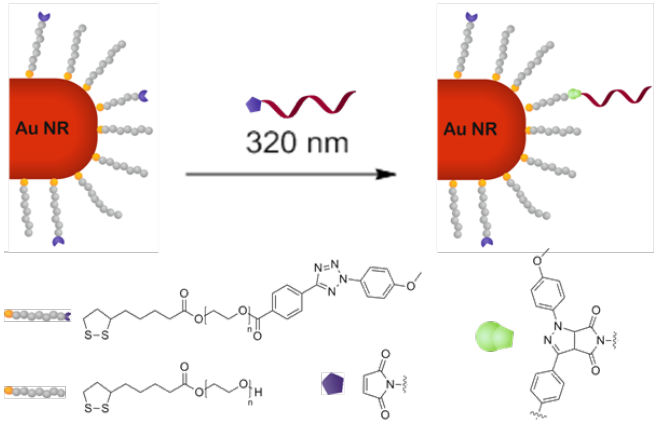


**Figure 1.** Light-induced biofunctionalization of Au NR with DNA. The novel photoreactive linker **1** and the inert linker **2** are attached to the Au NR surface. **1** enables the light-induced nitrile imine-mediated tetrazole-ene cycloaddition upon UV irradiation to generate the fluorescent pyrazoline product **3**.

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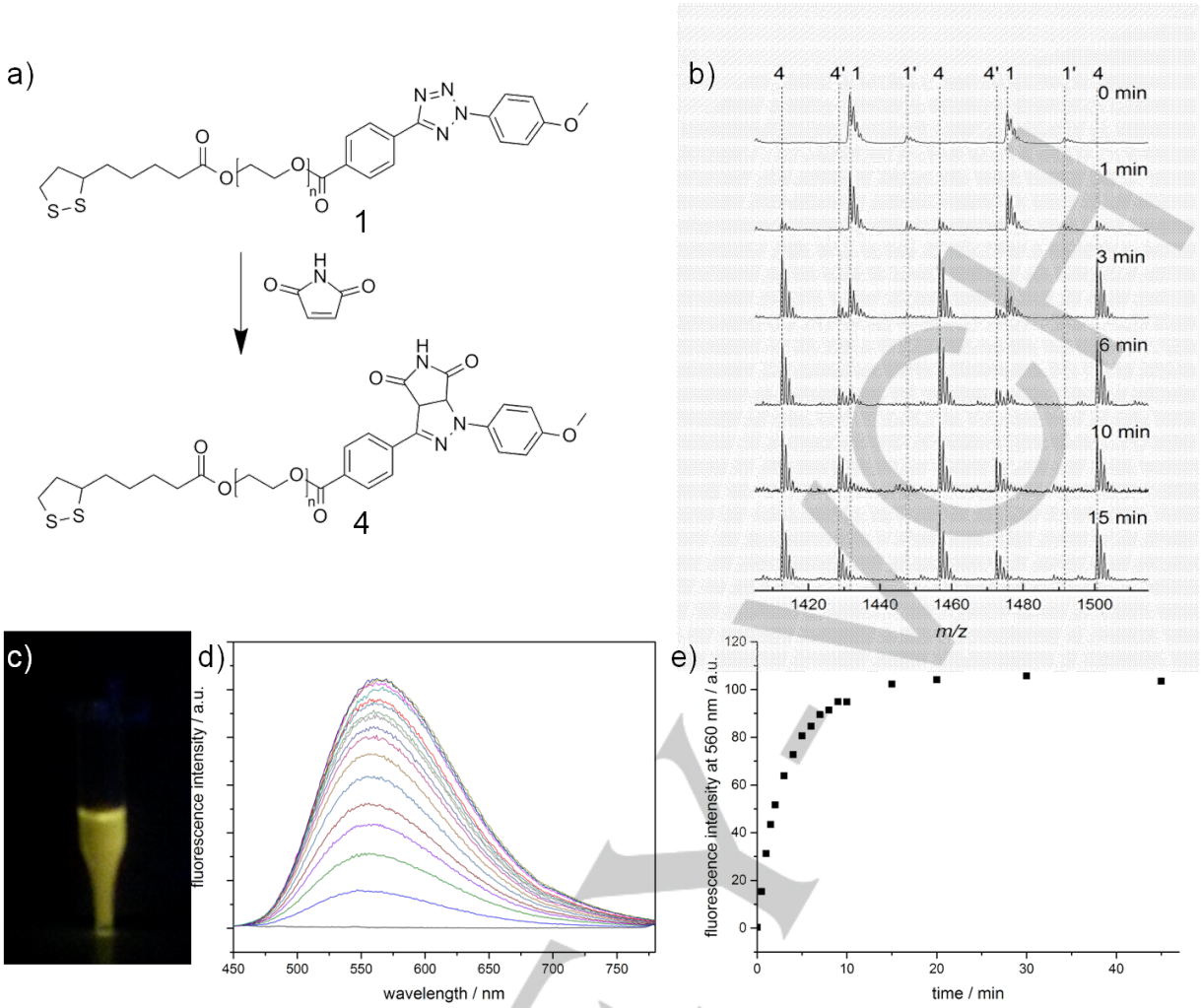


**Figure 2.** Results of the kinetic investigation of tetrazole based photoaddition of the model reaction. (a) Model reaction between the tetrazole linker **1** and maleimide. (b) ESI-MS spectra recorded after 0, 1, 3, 6, 10, 15 min depicts sodium adducts ($Na^+$) of **1** and **4**. **1'** and **4'** may be attributed to the oxidation during the ionization process. (c) Image of the vial containing fluorescent solution of the product **4**. (d) Fluorescence spectra of the reaction mixture ($\lambda_{ex}$ = 400 nm). (e) Evolution of the fluorescence signal at $\lambda_{em}$ = 560 nm derived from the fluorescence spectra.

thermal effect, have already shown promise for biomedical applications.[21]

One of the advantages of the NITEC reaction is the formation of a fluorescent pyrazoline product upon the reaction of the intermediate 1, 3-dipole, resulting from the release of the nitrogen from the initial tetrazole and the dipolarophiles of interest. Such reactions are particularly interesting for modification of NP surfaces, as they represent a self-reporting system in which the fluorescent product can directly be used for the assessment of the functionalization progress, which is often very difficult to achieve at nano-surfaces. NITEC has already been used for the generation of fluorescent polymers from non-fluorescent monomers,[22] the spatially resolved surface modification of various 2D substrates,[23] λ-orthogonal photoligation in combination with photoenol chemistry[16] and the generation of fluorescent single-chain polymeric nanoparticles.[24] There are also some examples for the rapid and selective modification of biomolecules for bioimaging, both *in vitro* and *in vivo*.[25] However, there is a gap in its use for nanomaterials modification and in their recent review, Medintz *et al.* mentioned such reactions as a missing link in nanoparticle modification.[2]

To achieve the biofunctionalization of nanorods by NITEC, we first designed an α,ω-bifunctional poly(ethylene glycol) (PEG) linker **1** ($M_n$ ~ 1500 g $mol^{-1}$), containing a tetrazole for further modification and a thioctic acid anchoring group for the attachment to the Au NR surfaces. Bidentate thioctic acid is known to provide a better colloidal stability to Au NPs than monodentate thiols and has been widely used for the attachment of various species to Au surfaces.[26] In addition, PEG is a commonly employed, water soluble and biocompatible polymer, which has been shown to enhance the stability of Au NRs in aqueous solutions and cell media.[27] Further, besides increasing the water stability of the NR, the long PEG chain can be considered as a separator between the NP core and the reactive tetrazole groups, decreasing the steric hindrance of the reaction. Furthermore, the distance between fluorophore (tetrazole reaction product) and NP surface is important to avoid fluorescence quenching and undesired fluorescence resonance energy transer (FRET) effects,[28] therefore creating an efficient self-reporting of system.

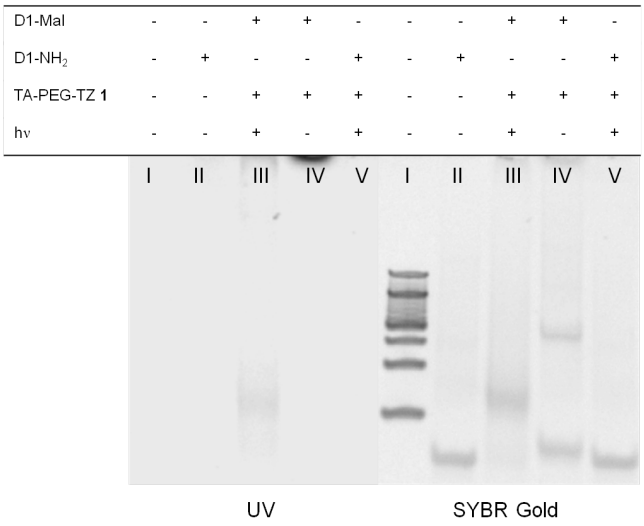


**Figure 3.** Gel electrophoresis of model reaction between TA-PEG-TZ **1** and Mal DNA without staining (left), with staining (right). Lane I: 10 bp marker; II: amino-DNA; III: model reaction of **1** and **D1-Mal**; IV+V: control experiments.

First, the reactivity of linker **1** was assessed by irradiation ($\lambda_{max}$ = 320 nm) of **1** and maleimide (Fig. 2a) in water/acetonitrile (9:1) and analyzed in detail by electrospray-ionization mass spectrometry (ESI-MS) showing complete conversion (Fig. 2b). Additionally, the reaction can be monitored by the resulting fluorescence of the pyrazoline product (Fig. 2c). The fluorescence spectra exhibit the typical broad, pyrazoline emission ($\lambda_{em, max}$ = 560 nm) at the excitation wavelength of $\lambda_{ex}$ = 400 nm (Fig. 2d). The reaction proceeds rapidly in the first few minutes when the nitrile imine concentration is high and subsequently plateaus after approx. 15 min (Fig. 2e). The quantum yield of the pyrazoline product was determined to be $\Phi_{fl}$ = 0.045, which is a slightly higher value than those of the comparable diphenyltetrazoles.[29]

After the successful assessment of the model reaction, tetrazole modified Au NRs-1 were prepared to enable the subsequent biofunctionalization. Au NRs (approx. 70 nm × 23 nm, aspect ratio 3) were synthesized according to a literature procedure.[30] The cetyltrimethylammonium bromide (CTAB) stabilized Au NRs were analyzed *via* UV/Vis spectroscopy and transmission electron microscopy (TEM). To attach the bifunctional linker **1** to the CTAB-coated Au NRs, a ligand exchange methodology was employed. Recently, a number of researchers have focused their efforts on the development of an efficient strategy to fully replace the CTAB with thiolated species, due to its toxicity and possible adverse effects in biomedical applications of Au NRs.[31] However, the removal of the CTAB surface stabilizers is not straightforward. We have followed several protocols adressing the ligand exchange of CTAB-capped Au NR with thiolated PEG in aqueous or ethanoic solution,[32] which either resulted in a low stability or agglomeration of the Au NRs, indicating a low efficiency of the ligand exchange. In general, the efficiency of the ligand exchange can be assessed by the stability of the Au NRs after dilution below the critical micelle concentration of CTAB[32a] and in the presence of salts. After several attempts to find a suitable ligand exchange procedure for our bifunctional linker **1**, we had success employing a phase transfer procedure adapted from Lista *et al.*[33] and a mixture of reactive linker **1** and inert PEG-thioctic acid linker **2** (in 1 : 10 ratio) in order to additionally minimize the surface crowding effects. The two phase system of Au NRs in aqueous solution and PEG linkers **1** and **2** in chloroform were vigorously stirred for 2 h after addition of 1M HCl solution. Upon successful phase transfer, the pegylated Au NRs-1 containing tetrazole moiety are dispersed in chloroform, centrifuged and redispersed in water. The efficient ligand exchange was confirmed by UV/Vis spectroscopy, indicating the presence of tetrazole at λ = 290 nm (Fig. S11), and the agarose gel, which showed the different mobilities of CTAB-capped Au NRs (Fig. 4c, lane I) and pegylated Au NRs-1 containing tetrazole (Fig. 4c, lane II). In addition, the 2.3 nm layer of PEG around the Au NR can be observed in HRTEM images, evidencing the expected size of the PEG linkers (Fig. 4a). UV-Vis studies (Fig. S11), TEM images (Fig. S12 and S13) as well as agarose gel studies indicate a high stability of Au NR after PEGylation and the photoreaction as no aggregation could be observed.

After the successful ligand exchange and displacement of CTAB, tetrazole modified Au NRs-1 were functionalized with maleimide containing DNA under irradiation. First, 5'-maleimide DNA (22mer, **D1-Mal**) was prepared using amino terminated ssDNA and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclo hexane-1-carboxylate (sSMCC) crosslinker. After purifciation by high-pressure liquid chromatography (HPLC), the reactivity of the maleimide modified ssDNA towards tetrazole was assessed by irradiation with the tetrazole containing linker **1**. Gel electrophoresis analysis indicated successful DNA-polymer conjugation only when both, linker **1** and **D1-Mal**, were present in the solution and irradiated (Fig. 3, lane III). Besides the lower mobility of the DNA-PEG conjugate due to its higher molecular weight, the formation of the product was additionally confirmed by the presence of the fluorescence band under UV irradiation.

Once the successful DNA-polymer conjugation was evidenced in solution, the tetrazole modified Au NRs-1 and **D1-Mal** were irradiated for 1 h to perform the DNA functionalization of Au NRs. The Au NRs were subsequently centrifuged and

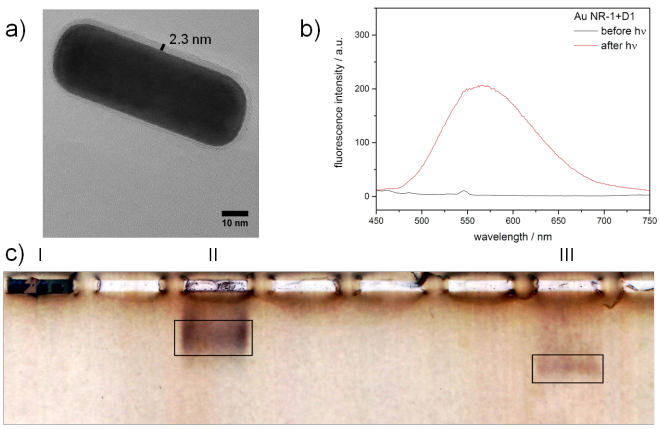


**Figure 4.** (a) HRTEM image of pegylated Au NR-1. (b) Fluorescence spectra before and after irradiation of the tetrazole-modified Au NR-1 and **D1-Mal**. (c) Agarose gel of CTAB-capped Au NR (lane I), pegylated Au NR-1 (lane II), and Au NR-DNA conjugate (lane III) indicating higher mobility of the DNA immobilized Au NRs.

thoroughly washed, after which the analysis was performed. Successful DNA immobilization on the Au NR was confirmed by a change in zeta potential (due to the negative charge on DNA, Table S3), which led also to a higher mobility in gel electrophoresis compared to unmodified Au NRs-1 (Fig. 4c). In addition, fluorescence measurements at an excitation wavelength of $\lambda_{ex}$ = 400 nm unambiguously confirmed the presence of the fluorescent pyrazoline product with a fluorescence emission at $\lambda_{em}$ = 565 nm after irradiation (Fig. 4b). Finally, to confirm that attached DNA remains fully functional after the immobilization, Au NRs-D1 was hybridized with complementary DNA strand **cD1.** Gel electrophoresis analysis showed that hybridization was successful (Fig. S13) allowing for the NRs to be further used in for i.e. origami decoration or the attachment of various species through DNA directed immobilization.[34]

In conclusion, we demonstrate the use of the light-induced tetrazole based NITEC reaction for biofunctionalization of gold nanorods. The novel bifunctional PEG linker containing thioctic acid as the Au anchoring group as well as the tetrazole moiety enabled the formation of fluorescent pyrazoline compounds within 1 h in aqueous solution. The fluorescent product can be used to assess the success of surface functionalization, representing an efficient self-reporting system. The system is particularly useful for monitoring nanoparticle surface modifications, which is usually challenging *via* standard analytical methods. We have shown that tetrazole Au NRs can be modified with DNA which can further be employed in hybridization protocols. The current first example of NITEC-mediated biofunctionalization of Au NR paves the way for the attachment of other sensitive biomolecules, such as antibodies and other proteins under mild conditions, offering the possibility of direct assessment of the reaction through formation of fluorescent product.

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**Keywords:** Au Nanorods • Photoinduced Dipolar Cycloaddition • DNA • Nanomaterial Functionalization

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# COMMUNICATION

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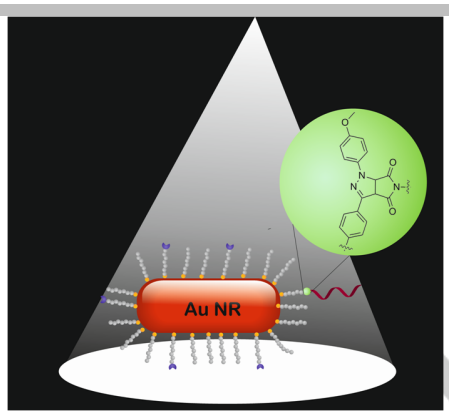


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