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Reversible Binding of Fluorescent Proteins at DNA–Gold Nanoparticles**

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Protein-functionalized gold nanoparticles (AuNPs) are currently attracting increasing interest as a result of their applications in the detection of antigens, which are specific biomarkers in disease diagnostics.^[1,2] Protein–AuNPs have long been known as versatile probes in immunohistochemistry and related techniques,^[3] while mixed hybrid particles containing both nucleic acid fragments and proteins have recently been established,^[4–7] because they offer additional functionality on account of the presence of both classes of biomolecular recognition elements. For instance, DNA–AuNPs containing a low number of antibody molecules have been successfully employed in the highly sensitive detection of a soluble pathogenic biomarker for Alzheimer's disease.^[6]

The synthesis of DNA–protein hybrid particles has been achieved by either chemisorption of the sulfhydryl groups of proteins to AuNPs followed by saturation of the particles with thiolated DNA oligonucleotides,^[5,6] or specific DNA hybridization using DNA–AuNPs and appropriate protein conjugates bearing the complementary DNA oligomer moiety.^[4,7] Herein, we report the extension of the latter approach to render the binding of DNA–protein conjugates to DNA–AuNPs reversible. To this end, we employed the concept of DNA oligonucleotide strand displacement, which has frequently been used in the development of nanomechanical DNA devices,^[8–14] and we have recently used this approach to reversibly switch the aggregation of DNA–AuNPs.^[15] The realization of the DNA-directed immobilization and detachment of proteins to gold nanoparticles, which is depicted schematically in Figure 3 a, would be advantageous over conventional chemisorption, because both immobilization and dissociation of the protein can be achieved under physiological conditions, thereby maintaining the full biological activity of the proteins.^[4] Moreover, this approach should

also open up ways to restore functional proteins from nanoparticle probes subsequent to their use in bioanalytical assays.

To experimentally investigate our hypothesis, we chose DNA conjugates of enhanced yellow fluorescent protein (EYFP), a mutant derivative of the naturally occurring green fluorescent protein from the North Pacific jellyfish *Aequorea victoria*, as a model system. This choice was made because the DNA–EYFP conjugate is considered to be a promising building block for the construction of biomimetic antennae and light-harvesting devices.^[16,17] We also expected it to be a convenient reporter because the fluorescence of the EYFP should be quenched upon binding to the gold nanoparticles, as previously reported for organic fluorescent dyes.^[18–24]

Initial experiments were carried out to investigate the binding of the covalent DNA–EYFP conjugate (**1** in Figure 1,

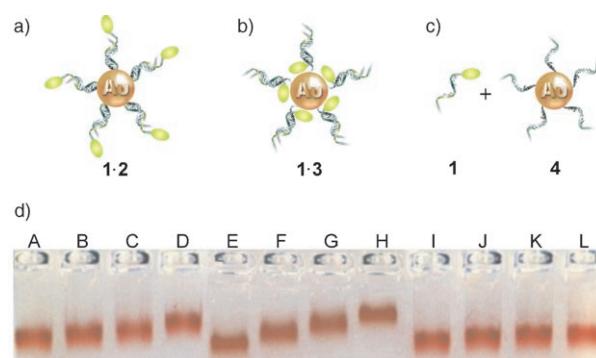


Figure 1. DNA–AuNPs **2** and **3** functionalized with EYFP conjugate **1** to form hybrid conjugates a) **1-2** and b) **1-3**, along with c) free conjugate **1** and noncomplementary DNA–AuNP **4**. d) Electrophoretic analysis of functionalization of gold nanoparticles with EYFP on a 1.5% agarose gel. The bands indicate the mobility of **2** (lane A), **2** coupled with 16, 32, and 64 molar equivalents of **1** (lanes B–D, respectively), **3** (lane E), **3** coupled with 16, 32, and 64 molar equivalents of **1** (lanes F–H, respectively), **4** (lane I), and **4** mixed with 16, 32, and 64 molar equivalents of **1** (lanes J–L, respectively).

EYFP-5'-AGC GGA TAA CAA TTT CAC ACA GGA-3') with DNA-coated 25-nm AuNPs **2-4**, which contain either complementary (**2**: AuNP-5'-TCC TGT GTG AAA TTG TTA TCC GCT-3', **3**: AuNP-3'-TCG CCT ATT GTT AAA GTG TGT CCT-5') or, as a control, noncomplementary DNA oligomers (**4**: AuNP-5'-TTT TTT TTT TTT GAT CCA GTA GAT A-3') as specific binding sites. Gel electrophoretic analysis was used to confirm the specific binding (Figure 1). In the case of DNA–AuNPs bearing complementary oligomers, which lead to conjugates **1-2** and **1-3**, a clear decrease in the electrophoretic mobility of the nanoparticles indicated the binding of the DNA–EYFP conjugate. In contrast, incubation of particles **4** with **1** only led to very small shifts of the respective bands (Figure 1 d, lanes J–L), thus confirming that the binding of **1** with **2** and **3** occurs primarily as a result of specific Watson–Crick base pairing of the oligonucleotides.^[25]

We then investigated whether the binding of **1** to the gold nanoparticle does indeed lead to quenching of the EYFP fluorescence (Figure 2). Fluorescence measurements were carried out with a solution of **1** (1.2 pmol in TETBS (Tris-HCl

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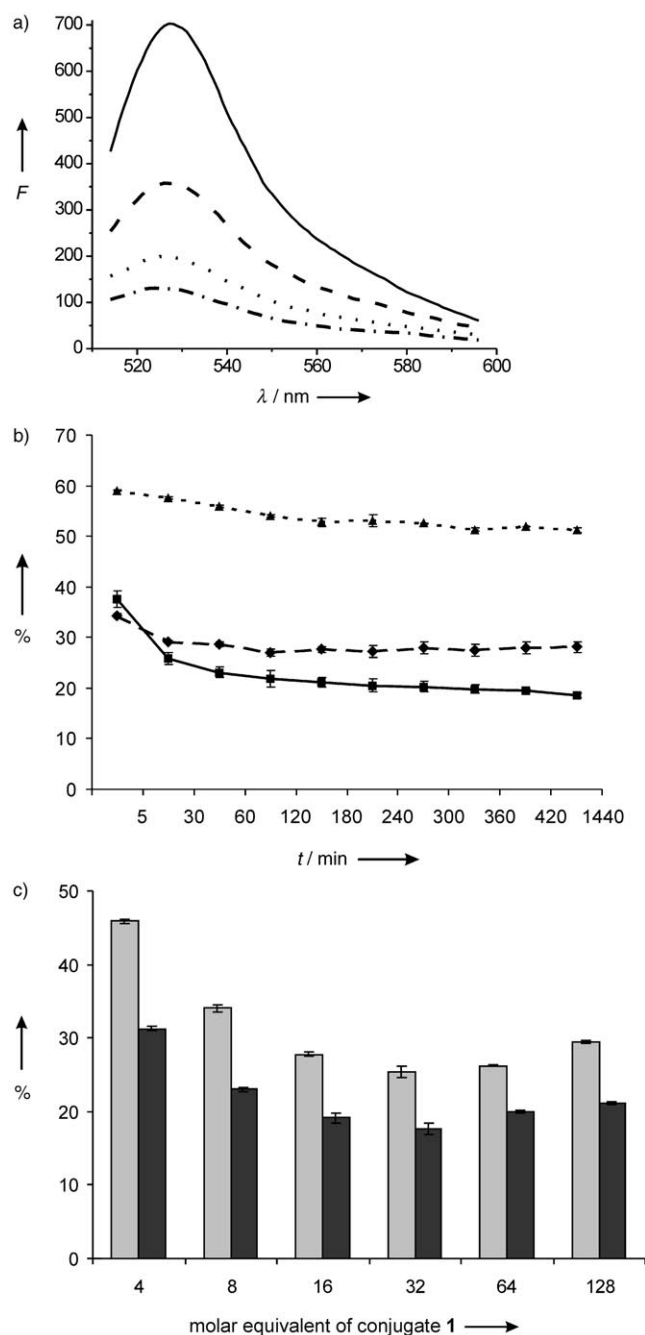


Figure 2. a) Fluorescence emission spectra of **1** (—), **1** mixed with **4** (----), **1** coupled with **2** (.....), and **1** coupled with **3** (-·-·-). b) Relative fluorescence of **1-2** (----), **1-3** (—), and **1-4** (.....) with respect to the fluorescence of **1** (100%) at 528 nm versus different incubation times. c) Relative fluorescence at 528 nm against various molar equivalents of **1** used for hybridization with DNA–AuNPs **2** (gray bars) and **3** (black bars). Note that the results obtained from end-point measurements are shown.

(20 mM), NaCl (150 mM), EDTA (5 mM), Tween (0.05%); pH 7.3; Tris = tris(hydroxymethyl)aminomethane) to which a solution of **2**, **3**, or **4** (36 fmol in TETBS buffer, corresponding to 32 molar equivalents of **1** to **2**, **3**, and **4**) was added. After incubation for 6 h, fluorescence spectra were recorded using the excitation wavelength $\lambda_{\text{exc}} = 490$ nm. As shown in Fig-

ure 2a, the addition of noncomplementary AuNPs **4** led to a decrease in fluorescence emission by about 50% as a result of strong absorption and scattering caused by the AuNPs. Addition of the complementary AuNPs, however, led to a significantly higher quenching of the EYFP fluorescence, and this effect was markedly different for particles **2** and **3**. In the case of **1-2**, where Watson–Crick hybridization should lead to the formation of a 24-base-pair double-stranded DNA (dsDNA) spacer approximately 8.5 nm in length between the protein and the gold surface, quenching was less efficient (approximately 44% of the fluorescence intensity of the respective control **1-4**) than in the case of conjugate **1-3** (approximately 64% of the fluorescence intensity of the control **1-4**), where hybridization should localize the protein in direct proximity to the particle's surface. The relatively small difference in quenching efficiency of only 20% observed for the two different configurations realized in **1-2** and **1-3** suggests that complex mechanisms form the basis of the quenching process.^[26]

The two different configurations in **1-2** and **1-3** (Figure 1) also affected the kinetics of protein immobilization (Figure 2b). Time-dependent fluorescence measurements showed that **1** rapidly hybridized with **2** and the reaction was completed within about 60 minutes. In contrast, the binding of **1** to **3** was significantly slower and led to a gradual decrease in relative fluorescence.^[27] The quenching reached a maximum after about 24 hours (data not shown), and consequently all the following experiments were carried out with incubation overnight. The slower binding of **3** is in agreement with the proposed configuration (see Figure 1b) because in the conjugate **1-3** the bulky EYFP needs to penetrate, at least partially, the dense layer of DNA oligomers attached to the surface of the particle.^[26]

We also studied the influence of surface coverage of AuNPs with EYFP on the amount of quenching (Figure 2c) by measuring the fluorescence emission intensities of **1**, and **2** and **3** coupled with various molar equivalents of **1**.^[27] The fluorescence decreased for the binding of up to about 32 molar equivalents of **1**, whereas a further increase of **1** led to an increase in fluorescence as a result of the increasing amount of free **1** in the solution. Hence, the data of Figure 2c indicate that about 32 molar equivalents of **1** can hybridize to 25-nm AuNPs, which correlates with an approximately 18% hybridization efficiency of the about 175 oligonucleotide molecules^[27] bound at the AuNP. This quantitative result is in very good agreement with earlier studies.^[28,29]

We also investigated whether the immobilized proteins can be restored from the gold nanoparticles. To this end, we synthesized DNA–AuNPs **5** and **6** (Figure 3a), which contain a four-nucleotide single-stranded overhang appended to the coding sequence (**5**: AuNP-5'-TCC TGT GTG AAA TTG TTA TCC GCT TACG-3', **6**: AuNP-3'-TCG CCT ATT GTT AAA GTG TGT CCT TACG-5'; the bases forming the dangling end are underlined). Hybridization of DNA–AuNPs **4**, **5**, and **6** with 32 molar equivalents of **1** led to quenching of the EYFP fluorescence in the case of **5** and **6**, similar to the case described above (light gray bars in Figure 3b). Then 1000 molar equivalents of the displacement oligomers **7** (corresponding to **1-5**; 5'-CGT AAG CGG ATA ACA ATT

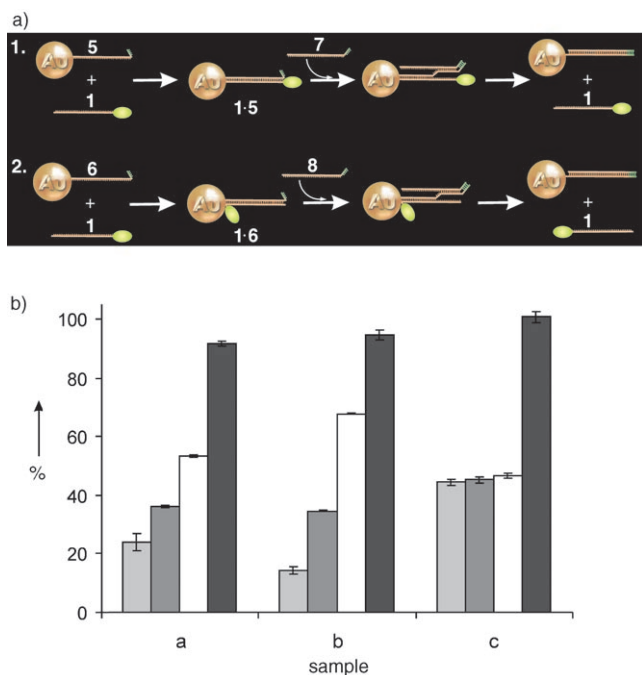


Figure 3. a) DNA-directed binding and detachment of DNA–AuNPs with/from DNA–EYFP conjugate **1**. DNA–AuNPs **5** or **6** were functionalized with EYFP conjugate **1** through specific nucleic acid hybridization, and subsequently strand-displacement oligonucleotides **7** and **8** were added to the conjugates **1-5** and **1-6**, respectively, which led to the formation of dsDNA-functionalized nanoparticles and regeneration of **1**. b) Quenching and regeneration of the EYFP fluorescence. The relative fluorescence values at 528 nm are shown. The light-gray bars represent the fluorescence of **1** in the presence of **5** (sample a), **6** (sample b), or **4** (sample c). The dark-gray bars represent the increased fluorescence of conjugate **1** upon addition of strand-displacement oligomer **7** (samples a and c) or **8** (sample b). The white bars represent the further increase in fluorescence upon increase of the NaCl concentration to 0.5 M within samples a–c. The black bars represent the fluorescence obtained after treatment of **1-5** (sample a), **1-6** (sample b), and unhybridized **4** (sample c) with mercaptoethanol. Note that 100% represents the fluorescence of the original solution containing **1**.

TCA CAC AGG A-3') or **8** (corresponding to **1-6**; 5'-AGC GGA TAA CAA TTT CAC ACA GGA ATG C-3'), complementary to DNA–AuNPs **5** and **6**, respectively, were added and the mixture was incubated overnight. As shown in Figure 3b (dark gray bars), the restoration of the fluorescence was clearly observable in the case of particles **5** and **6**, and this led to almost identical fluorescence intensities compared with that of the control reaction containing the noncomplementary particles **4**. Further variations of the amount of **7** or **8** indicated that 500 molar equivalents led to restoration of about 75% of the control value, while 250 equivalents led to restoration of about 70% (data not shown). Taking into account the surface coverage of the AuNPs (about 175 oligonucleotides/particle), these data suggest that an approximately sixfold excess of the displacement oligomer with respect to the AuNP-bound DNA is necessary to effectively drive the equilibrium toward complete dissociation of the DNA–EYFP conjugate.

To verify that the decrease in fluorescence observed upon mere addition of AuNPs is a result of bulk effects, we studied whether the dsDNA–AuNPs formed upon strand displacement might be precipitated by an increase in salt concentration. This phenomenon was recently observed and elaborated by Maeda and co-workers.^[30–32] Indeed, increasing the salt concentration of the reaction samples, prepared as described above, to 0.5 M NaCl led to immediate precipitation of the AuNPs within about 15 minutes in the case of **5** and **6**, as could be clearly observed by naked-eye inspection of the samples. Fluorescence measurements of these samples confirmed that the fluorescence after particle precipitation was higher (white bars in Figure 3b) than in the presence of the dispersed particles (dark gray bars in Figure 3b); however, complete restoration of the original EYFP fluorescence was not observed in any of the samples.

As an alternative to the liberation of the EYFP by DNA strand displacement, we also tested whether it is possible to restore the protein by complete stripping of the particle-attached DNA by treatment with mercaptoethanol.^[28] Naked-eye observation indicated that here precipitation occurred significantly slower than in the experiments described above (about 4 h). As shown in Figure 3b (black bars), the addition of mercaptoethanol (12 mM final concentration) led to almost complete restoration of the initial EYFP fluorescence. Thus, fluorescence almost equal to that of the original EYFP solution (that is, 100%) was observed, which indicates that quenching was not persistent in the AuNP-containing samples after precipitation. Although the liberated DNA–protein conjugate now contains dsDNA, this approach might be well-suited for applications in which the DNA–AuNP-bound protein, for instance, comprises antibody or receptor moieties to specifically capture a target molecule from a complex biological matrix.

In conclusion, we have shown that proteins can be reversibly bound to DNA–gold nanoparticles without compromising their biological activity. The use of fluorescent proteins also demonstrates for the first time that these biological fluorophores can be incorporated into nanoparticle-based devices, thus leading to optically coupled hybrid architectures. The utilization of this system would also allow a systematic study of distance-dependent fluorescence quenching to be carried out by simply varying the lengths of the oligonucleotides attached to protein and the AuNPs. Further optimization of these approaches with respect to experimental parameters, such as temperature, salt concentration, and pH, by using oligofunctional DNA–nanoparticles^[29] and, in particular, by design of appropriate oligomer sequences^[14] and recombinant fluorescent protein mutants, should pave the way toward nanostructured materials with programmable functionalities for a broad range of applications in nanobiotechnology.

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- [25] EYFP has an isoelectric point of about 6.2, and thus its slightly negative net charge at pH 7.3 should prevent the nonspecific binding of **1** to the negatively charged DNA–AuNPs. The small degree of binding observed in gel electrophoresis might be a result of Van der Waals interaction, which was previously observed in EYFP bioconjugation.^[16]
- [26] The fundamentals of the quenching processes occurring in organic fluorophores attached to the surface of metal particles are still under investigation.^[19–24] AuNPs have been shown to quench organic fluorophores by decreasing their radiative rate and increasing their nonradiative rate,^[19] and the energy transfer has been shown to follow a distance dependence that is proportional to $1/r^4$ ^[21] rather than the Förster-type $1/r^6$ proportional transfer efficiency dependence observed for fluorescent quantum dots on the surface of AuNPs.^[20] In contrast, the enhancement in fluorescence has also been reported for organic fluorophores in the vicinity of AuNP surfaces.^[24] A crucial aspect in all these studies concerns the correct determination of the distance between the chromophore and the metal surface. In the case reported here, the NP-bound DNA should be able to adopt a number of different conformations, such as being fully stretched and pointing perpendicularly from the surface, adopt-
- ing a random coil shape, or perhaps being tilted and interacting with the surface of the AuNP (W. J. Parak, T. Pellegrino, C. M. Micheel, D. Gerion, S. C. Williams, A. P. Alivisatos, *Nano Lett.* **2003**, *3*, 33–36). Hence, the nominal difference in distance of about 8.5 nm for assemblies **1-2** and **1-3** is likely to be affected by the presence of various conformational states, and therefore this might contribute to the explanation of the similarity in fluorescence quenching observed. Moreover, slight changes in protein loading should also induce different distances and/or different dynamics, and thus might also affect the quenching efficiency.
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