ABSTRACT

We report the first homogeneous sandwich immunoassay with gold nanoparticles (AuNPs) as fluorescence quenchers. The sandwich assay is designed for the detection of the protein cardiac troponin T (cTnT) by its simultaneous interaction with two different antibodies, one attached to AuNPs and the other labeled with fluorescent dyes. We demonstrate the working principle of the assay and using time-resolved fluorescence spectroscopy, we determine the quenching efficiency of the gold nanoparticles. In spite of the relatively large separation distance between dye molecules and AuNPs, ranging from 3 to 22 nm, the AuNPs quench the fluorescence with efficiencies as high as 95%. A limit of detection of 0.02 nM (0.7 ng/mL) was obtained for cTnT, which is the lowest value reported for a homogeneous sandwich assay for cTnT. These results illustrate the use of metallic nanoparticles as fluorescence quenchers in immunoassays where the large biomolecules involved impose distances for which energy transfer between fluorophores would be inefficient.

Imunoassays are commonly used for the detection of analytes, for example, proteins, by their specific interaction with antibodies. Sandwich immunoassays, where two antibodies bind to two different specific recognition sites (epitopes) on a protein, are of great interest because they provide high sensitivities and do not need manipulation of the analyte solution prior to the test. Förster resonance energy transfer (FRET) from donor to acceptor fluorescent labels attached to these antibodies can take place when the sandwich is assembled.\(^1\) The efficiency of FRET depends strongly on the relative distance and orientation of the donor and acceptor dipole. For optimum (parallel) donor—acceptor orientation, practical FRET efficiencies between fluorescent molecules are typically limited to donor—acceptor distances smaller than $5—7$ nm. Since the size of protein—antibody complexes may reach $10$ nm or more, the efficacy of these sandwich fluoroimmunoassays is limited. Assay performances have been improved by using quantum dots\(^2\) or phosphorescent dyes\(^3\) as donors or special antibody fragments designed to arrest donor and acceptor units in close proximity once binding to the protein has occurred.\(^4\) Here, we demonstrate the use of gold nanoparticles as highly efficient long-range fluorescence quenchers (acceptors) in sandwich immunoassays.

The reason why gold nanoparticles (AuNPs) are highly efficient fluorescence quenchers over longer distances\(^5—7\) is manifold. First, since the spherical AuNPs have no defined dipole moment as dye molecules do, energy transfer (ET) to the AuNP takes place for any orientation of the donor relative to the surface of the AuNPs. Second, due to the size of the AuNPs, the ET efficiency from a molecular donor to an AuNP acceptor decays slower with distance as compared to the case of a molecular acceptor. In addition, the large absorption cross section of AuNPs, especially near their plasmon resonance, enhances their performance as energy acceptors. Finally, in contrast to molecular acceptors, AuNPs may also quench fluorescence due to radiative rate suppression.\(^8—9\) Recently, fluorescence quenching by AuNPs has been used in immunosensors. However, an additional washing step was required,\(^10\) magnetic beads have been utilized,\(^11\) conformational changes of antibodies were used,\(^12\) or a competitive format was applied.\(^13\) Biotin assays with sensitivities in
the micromolar range have also been reported. We make use of the long-range fluorescence quenching by AuNPs in a sandwich type immunoassay for the detection of the medically relevant protein cardiac troponin T and achieve a limit of detection in the picomolar range. Using time-resolved fluorescence spectroscopy we are able to separate the contributions of bound and unbound labeled antibodies. This allows us to understand in detail the mechanism of the fluorescence quenching process and to identify all the parameters of the assay which influence the quenching efficiency.

Materials and Methods. Components of the Assay. Cardiac troponin T (cTnT) is a protein released into the bloodstream upon heart muscle damage and its testing is done to diagnose heart attacks (myocardial infarctions). cTnT contains 288 amino acids and has a molecular weight of 37 kDa. We used bivalent antibody fragments F(ab′)2 of the monoclonal antibodies M7 and M11.7 that bind to two different locations on the cTnT protein, namely to the amino acids 125–131 and 136–147, respectively.

The surface of AuNPs with a diameter of 20 nm was functionalized with anti-cTnT M11.7 fragments according to a method described in ref 18. The average number of active anti-cTnT M11.7 binding sites on each AuNP is estimated to be 40. The remaining free surface was passivated with bovine serum albumin (BSA).

Cy3 and Cy3B (GE Healthcare) fluorophores were attached to anti-cTnT M7 according to the following procedure. The M7 antibody was dissolved in phosphate buffered saline (PBS) and a controlled amount of N-hydroxysuccinimide-activated dye molecules was added. The solution was slowly shaken under protection from light and the reaction was stopped by the addition of lysine. This resulted in the covalent attachment of dye molecules to lysine amino acids of the antibody through stable peptide bonds. The degree of antibody labeling was controlled by tuning the concentration of the activated fluorophore and the reaction time. The degree of labeling was determined by absorption measurements at the corresponding dye absorption peak and at the antibody absorption at 280 nm. In the case of M7-Cy3 there were 5.4 ± 0.3 dye molecules incorporated per antibody and 2.4 ± 0.2 in the case of M7-Cy3B. The quantum efficiencies of the M7-Cy3 and M7-Cy3B conjugates were determined to be 0.04 and 0.12, respectively.

In the following, we refer to the antibody-dye conjugate as M7-dye and to the antibody-AuNP conjugate as M11.7-AuNP. The concentrations of these complexes are denoted as [M7-dye] and [M11.7-AuNP].

The emission spectra of Cy3 and Cy3B fluorophores have sufficient overlap with the absorption spectra of AuNPs, which is a prerequisite for energy transfer from dye to AuNP (Figure 1). The dye emission peaks also lie close to the plasmon resonance that provides for a longer distance range for ET. The comparison of Cy3 and Cy3B allows us to investigate experimentally the impact of different de-excitation dynamics of the donor on the quenching by the AuNPs.

We used tris(hydroxymethyl)aminomethane buffer (Trizma pH 7.5, Sigma) in 20 mM concentration with additional 1 mg/mL of bovine serum albumin. The analyte solutions consisted of cTnT in lyophilized human serum. The final serum concentration introduced into the tests by the analyte solution was 2%.

Steady state fluorescence measurements were performed with a Fluorolog-3 spectrophotometer (Horiba Jobin Yvon) at the excitation wavelength of 520 nm. Time-resolved measurements were performed with a streak camera (Hamamatsu C5680). Pulsed (150 fs fwhm, 75.6 MHz repetition rate) excitation at 545 nm was provided by an optical parametric oscillator (APE Berlin) pumped with a Ti:sapphire laser (Mira, Coherent).

Principle of Operation and Assay Sensitivity. The principle of operation of the sandwich test for cTnT. AuNPs are functionalized with anti-cTnT M11.7 antibody fragments. Anti-cTnT M7 antibody fragments are labeled with fluorescent dye molecules. Upon addition of cTnT, the M11.7-AuNP and the M7-dye bind to different positions of the cTnT molecules forming sandwich assemblies. As a result, the fluorescence of the dye is quenched by the nearby AuNP.

The number of sandwich assemblies formed depends on the concentrations [M11.7-AuNP], [M7-dye], and [cTnT] and
on the binding affinity of the assay components. A characteristic feature of homogeneous sandwich tests is the high dose hook effect.\textsuperscript{20,21} As \([\text{cTnT}]\) increases, the number of sandwich complexes first increases, reaches a maximum, and then decreases again. Consequently, the fluorescence intensity decreases, reaches a minimum, and then increases again (Figure 3A). This recovery of fluorescence occurs because the \([\text{cTnT}]\) is so high that most of the M7-dye or M11.7-AuNP bind to individual cTnT molecules and do not form sandwiches. The actual shape of the curve describing the fluorescence emission versus \([\text{cTnT}]\) is determined by the concentrations of AuNP-conjugated and dye-labeled antibodies, and their respective affinity constants for the binding to cTnT. For an unambiguous detection and quantification, it is necessary to work in the initial analyte concentration range before the minimum in fluorescence is reached. To maximize the number of sandwich assemblies while minimizing the number of free M7-dye (fluorescence background), the concentrations should fulfill \([\text{M11.7-AuNP}] > [\text{cTnT}] > [\text{M7-dye}]\). Then, reducing the concentration of dye-labeled antibody leads to a more sensitive assay (Figure 3B). In practice, the minimum functional \([\text{M7-dye}]\) may be limited by the photodetectors or the fluorescence background of the sample. Up to a certain value, increasing the \([\text{M11.7-AuNP}]\) leads to saturation at higher \([\text{cTnT}]\); that is, it increases the dynamic range of the assay. Beyond this point, higher \([\text{M11.7-AuNP}]\) delays the fluorescence recovery. However, the \([\text{M11.7-AuNP}]\) cannot be arbitrarily high because of fluorescence reabsorption, which becomes important at the low \([\text{M7-dye}]\) conditions necessary for a highly sensitive assay.

For the concentrations of M11.7-AuNP and M7-Cy3B shown in Figure 3B we obtained a 3σ limit of detection\textsuperscript{22} of 0.02 nM (0.7 ng/mL). It should be noted that this limit is set by the variance in the fluorescence intensity of the blank samples, which is in our case determined by pipetting errors. In a comparative measurement to the blank fluorescence signal, smaller quantities may be readily detected as demonstrated in the example shown in Figure 3B, where 0.017 nM of cTnT produces a 30% reduction of the fluorescence signal. The sensitivity of our assay lies in the diagnostically relevant range and is to our knowledge the best value reported for a cTnT homogeneous sandwich assay. Higher sensitivities have been achieved with heterogeneous immunoassays involving washing and/or separation steps.\textsuperscript{23}

**Geometric Considerations.** The distance between the dyes and AuNPs in the sandwich assemblies can be estimated from the sizes of the components. Each F(ab')\textsubscript{2} fragment consists of two Fab' fragments that are approximately 5 nm long\textsuperscript{24,25} and are flexibly connected at one end. On the basis of the molecular weight, we estimate a minimum size of cTnT of roughly 2.5 nm in each dimension. The Cy3 and Cy3B molecules were attached to lysine amino acids situated at various locations on the surface of the M7-F(ab')\textsubscript{2} fragments. The M11.7-F(ab')\textsubscript{2} fragments were adsorbed onto the surface of the AuNPs with no preferential orientation. In addition, BSA molecules, which have dimensions of about 8.4 nm × 8.4 nm × 3.2 nm\textsuperscript{26} are adsorbed to the AuNPs surfaces. It follows that the BSA sets a lowest limit of roughly 3.2 nm for the distance between the AuNP and the dye molecules. On the other hand, the maximum achievable dye-AuNP distance is of 22.5 nm and corresponds to a situation where the M11.7-TnT-M7 complex is fully extended and perpendicular to the surface of the AuNP. These two extreme situations set the range for the possible AuNP-dye distance boundaries but probably intermediate situations are the most frequent ones.

The flexibility of antibodies and the fact that there is more than one dye molecule per antibody lead to an average random orientation of the dye transition dipoles with respect to the AuNP surface.

**Time-Resolved Fluorescence Analysis.** The overall quenching response of the immunoassay is given by the product of the number of sandwich assemblies formed and the quenching efficiency. These two contributions can be separated by analyzing the time-resolved fluorescence of the M7-dye conjugates and of the AuNP-M11.7-cTnT-M7-dye sandwich assemblies.

In Figure 4, fluorescence decays of the unbound M7-Cy3 and M7-Cy3B are shown (i.e., for \([\text{cTnT}] = 0\)). Additionally, the respective fluorescence decays of the sandwich assemblies are shown as well (i.e., for \([\text{cTnT}]\) near the fluorescence minimum, see Figure 3A). The formation of the sandwich leads to a faster de-excitation of both fluorophores indicating the presence of energy transfer to the AuNPs.

![Figure 3](image-url)
Figure 4. Time-resolved response of the immunoassay. Fluorescence decays of the unbound M7-dye in solution (red and green) and in the sandwich assembly (gray) for Cy3 (A) and Cy3B (B). In (A,B), the fluorescence intensities are normalized to the maximum emission of the respective unbound M7-dye solutions. [M11.7-AuNP] = 0.25 nM, [M7-dye] = 2.50 nM, [cTnT] = 2 nM. The black lines are the results of rate equation modeling. The obtained decay times of the fast (τf) and slow (τs) components for the M7-dye and the sandwich assembly (τ'f and τ's) are shown above the graphs.

Although free Cy3 and Cy3B present a single-exponential fluorescence decay (not shown), they show a more complex behavior when they are bound to the M7 antibodies. This modified photophysics is attributed to two main effects. The first effect is quenching of fluorescence by tryptophan. The dye molecules are randomly attached to a number of possible lysine residues on the M7 and will thus present a distribution of distances to tryptophan residues. This quenching is expected to be weak for Cy3 but of importance for Cy3B. The second effect is the conformational stabilization of the dyes upon binding to the M7. It is known that Cy3 may present a longer fluorescence lifetime when chemically conjugated to other molecules.

The fluorescence decays of free M7-Cy3 and M7-Cy3B (i.e., [cTnT] = 0) can be satisfactorily described by a double exponential \( I(t) = A_F e^{-\frac{t}{\tau_F}} + A_S e^{-\frac{t}{\tau_S}} \) (Figure 4). We therefore describe each M7-dye system as composed of two subensembles, a fast one with decay time \( \tau_F \) and a slow one with decay time \( \tau_S \). By fitting rate equation calculations to the experimental fluorescence decays we determine \( \tau_F, \tau_S, A_F, \) and \( A_S \) (Figure 4). For each subensemble, the amplitudes \( A_F \) or \( A_S \) are given by the product of the number of dye molecules and their radiative rate. We define \( R = A_S/A_F \) as the ratio of the amplitudes.

When cTnT is added and the sandwich assemblies are formed, the influence of the nearby AuNPs on each of the subensembles of M7-dye needs to be considered. Therefore, the fluorescence decay of the system for [cTnT] ≠ 0 needs to be modeled with a 4-fold exponential in order to account for the free M7-dye and for the M7-dye quenched by the AuNPs in the sandwich assembly

\[
I(t) = A_{FF} e^{-\frac{t}{\tau_F}} + A_{FS} e^{-\frac{t}{\tau_S}} + A_{SF} e^{-\frac{t}{\tau_F}} + A_{SS} e^{-\frac{t}{\tau_S}}
\]

where \( A_{FF}, A_{FS}, \tau_F, \) and \( \tau_S \) describe the M7-dye complexes that stay free in the solution and \( A_{SF}, A_{SS}, \tau_F, \) and \( \tau_S \) describe the dye molecules quenched by AuNPs in the sandwich assemblies. The sum of all amplitudes equals to the total fluorescence intensity at time zero, \( A_{FS} + A_{SF} + A_{SS} + A_{FF} = I(0) \). It is found that upon addition of cTnT, the overall reduction of \( I(0) \) is less than 10% (Figure 4, Table 1). This indicates that the radiative rates of the dyes in the M7-dye conjugates do not change considerably upon cTnT binding.

**Table 1. Change in the Intensity at Time Zero and Energy Transfer Rates for the Slow and Fast Components of the M7-Cy3 and M7-Cy3B Markers**

<table>
<thead>
<tr>
<th></th>
<th>( \Delta I(0) )</th>
<th>( \Phi_{ET,F} )</th>
<th>( \Phi_{ET,S} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>M7-Cy3</td>
<td>(4±2)%</td>
<td>(73±1)%</td>
<td>(62±1)%</td>
</tr>
<tr>
<td>M7-Cy3B</td>
<td>(7±2)%</td>
<td>(95±1)%</td>
<td>(81±1)%</td>
</tr>
</tbody>
</table>

and therefore, permits to consider to a good approximation that \( A_S = R A_F \) and \( A_S = R A_F \). This leads to the following dependency: \( A_{FF} = I(0) / (1 + R) - A_F \). Then, the decays for the samples containing sandwich assemblies (i.e., [cTnT] ≠ 0) are fitted with

\[
I(t) = A_{FF} e^{-\frac{t}{\tau_F}} + RA_F e^{-\frac{t}{\tau_S}} + \left[ \frac{I(0)}{1 + R} - A_F \right] e^{-\frac{t}{\tau_F}} + R \left[ \frac{R(0)}{1 + R} - A_F e^{-\frac{t}{\tau_S}} \right]
\]

where \( R, \tau_F, \) and \( \tau_S \) are known from the fits to the decay in the absence of cTnT and only \( A_F, \tau_F, \) and \( \tau_S \) are the free parameters (Figure 4).

From this analysis, we first obtain the fraction of M7-dye forming sandwich assemblies which is given by \( 1 - A_{FF} / A_F \). where \( A_F \) and \( A_{FF} \) are obtained from respective measurements at [cTnT] = 0 and [cTnT] ≠ 0, and identical concentrations [M11.7-AuNP] and [M7-dye]. We find that the fraction of M7-Cy3B forming sandwich assemblies is higher than that of M7-Cy3. For [M11.7-AuNP] = 0.25 nM, [M7-dye] = 2.50 nM, and [cTnT] = 2.00 nM, the fraction of M7-Cy3 forming sandwich is (68 ± 5)% for M7-Cy3 and (76 ± 4)% for M7-Cy3B. This difference in affinity of the two M7-dye conjugates to cTnT is attributed to the different degrees of labeling. There are in average twice as many dye molecules per M7 antibody in the case of the M7-Cy3 compared to M7-Cy3B. Some of those Cy3 molecules may be attached to the M7 in positions that obstruct the binding to cTnT. Thus, time-resolved spectroscopy enables the determination of the fraction of the dye-labeled antibodies bound to form sandwich complexes. Investigations like this one at different concentrations of the assay components would enable the determination of the affinity constants of M7 and M11.7 to cTnT. However, the main focus of this article is the investigation of the fluorescence quenching by the AuNPs and not that of the binding affinity.
The fluorescence quenching is a result of the interplay between radiative rate modifications and energy transfer. The nearby AuNPs can lead either to enhancement or to suppression of the radiative rate of the dye molecules depending on the distance and orientation of the molecular transition dipole to the surface of the AuNP. For the dye-AuNP distances involved in our experiments, dye molecules with their transition dipole parallel (perpendicular) to the AuNP surface have a suppressed (enhanced) radiative rate. As discussed above, the net modifications of the radiative rate are less than 10%. The small contribution to the overall fluorescence quenching is probably due to the random location and orientation of the dye molecules with respect to the surface of the AuNP.

Therefore, the strong reduction of fluorescence intensity experienced by the fluorophores when bound to AuNPs in the sandwich assemblies is mainly caused by energy transfer. For each of the subensembles of dye molecules (fast or slow), the efficiency of the ET is given by

\[ \Phi_{\text{ET}} = \frac{\Gamma_{\text{ET}}}{\Gamma_{\text{ET}} + \Gamma_{\text{nr}} + \Gamma_r} \]  

(3)

where \( \Gamma_{\text{ET}} \) is the energy transfer rate, \( \Gamma_{\text{nr}} \) is the nonradiative rate, and \( \Gamma_r \) is the radiative rate. It is evident that small variations in \( \Gamma_r \) lead to even smaller variation in \( \Phi_{\text{ET}} \), in particular when \( \Phi_{\text{ET}} \) is large. The observed \( \Gamma_r \) variations of around 10% allow us to calculate the ET efficiency as \( \Phi_{\text{ET}} = 1 - \tau/\tau_r \) for the slow and fast subensembles (Table 1). The high values of \( \Phi_{\text{ET}} \) obtained validate our calculation.

In comparison to Cy3, the more efficient Cy3B presents values of \( \Phi_{\text{ET}} \) that are about 15% higher. The reason for this is the direct proportionality between \( \Gamma_{\text{ET}} \) and \( \Gamma_r \), which involves two processes with a similar value of \( \Gamma_r \). We write this proportionality as \( \Gamma_{\text{ET}} = \alpha \Gamma_r \), and recall that the fluorescence quantum efficiency is \( \Phi_F = \Gamma_r/(\Gamma_r + \Gamma_{\text{nr}}) \). It is straightforward to see that there is a positive correlation between fluorescence quantum efficiency and energy transfer efficiency

\[ \Phi_{\text{ET}} = \frac{\alpha \Phi_F}{\alpha \Phi_F + 1} \]  

(4)

Therefore, higher ET efficiencies should be achieved by using antibody-dye complexes with higher fluorescence quantum yields. This expression also explains the behavior of the fast subensembles. Those molecules present faster nonradiative decays that lead to a lower quantum efficiency and a consequently lower energy transfer efficiency.

Another factor that would lead to higher ET efficiencies is a better overlap of the dye emission spectrum and the absorption (plasmon) spectrum of the AuNPs. Last but not least, controlling the dye labeling of the antibody could further improve the assay performance. A parallel orientation of the dye transition dipole moment to the surface of the AuNP would be of benefit. Additionally, as it could be seen, the degree of labeling of antibodies with dye should be chosen carefully so that dye molecules do not obstruct the binding site and hence do not interfere with the antigen—antibody binding reaction.

Conclusions. We report the first homogeneous sandwich immunoassay with AuNPs as fluorescence quenchers. The sandwich assay is designed for the detection of cTnT by its simultaneous interaction with M11.7 antibodies attached to AuNPs and with M7 antibodies labeled with fluorescent dyes. We compared the performance of two M7-dye conjugates each having a different dye molecule and degree of labeling. Using time-resolved fluorescence spectroscopy, we separate the contributions of the protein-antibody binding affinities and the quenching efficiency to the overall quenching signal.

Despite the relatively large separation distance between dye molecules and AuNPs, ranging from 3 to 22 nm, the AuNPs quench the fluorescence effectively. Because of the random location and orientation of the dye labels, variations of the radiative decay rate are averaged out and produce only a minor contribution to the fluorescence quenching. The strong fluorescence quenching is caused by ET from the excited dye molecules to the AuNP, which occurs with efficiencies as high as 95%.

A limit of detection of 0.02 nM (0.7 ng/mL) was determined, which is the highest value reported for a cTnT homogeneous sandwich assay. The assay sensitivity is inversely proportional to the concentration of M7-dye. Therefore, the assay sensitivity is limited in this case by the fluorescence background. Although a higher sensitivity is expected by using multiple dye labels on each M7, we find that it leads to a decreased M7-cTnT binding affinity, presumably because some of the dye labels are placed inconveniently near the cTnT paratope of the M7. The best way to improve the assay sensitivity is to (a) optimize the spectral overlap of the dye emission with the plasmon resonance of the nanoparticles and (b) use highly efficient fluorophores because it simultaneously enables the use of lower concentrations of M7-dye and produces a higher efficiency of ET. The concepts discussed here are also applicable to other assay designs, that is, using other antibodies, analytes, fluorescent dyes, and even other metallic nanoparticles.

Acknowledgment. We gratefully acknowledge support by the Bayerische Forschungsförderung and by the DFG through the Excellence Cluster “Nanosystems Initiative Munich” (NIM). S.M. is thankful for the support by the International Doctoral Program “NanoBioTechnology” funded by the Elitenetzwerk Bayern.

References