DNA detection assay based on fluorescence quenching of rhodamine B by gold nanoparticles: The optical mechanisms

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DNA detection assay based on fluorescence quenching of rhodamine B by gold nanoparticles: The optical mechanisms

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1. Introduction

The conventional strategy for nucleic acid detection is based on the polymerase chain reaction (PCR) [1], which allows quantitative DNA determination with high specificity, sensitivity, and efficiency and with low contamination risk [2]. Despite its almost routine applications, the PCR method requires special reagents and cost-effective equipment. Therefore, in certain circumstances (e.g., in warfare or under field conditions), it is desirable to have a rapid and simple qualitative test for primary DNA detection to compete with precise PCR analysis. Colloidal gold nanoparticles (GNPs) form a new platform for colorimetric DNA detection [3,4]. The change in solution color and the plasmon resonance shift that occur owing to particle aggregation are used in various tests for DNA detection [5–9]. The dynamic light scattering detection of DNA has been proven to be more sensitive than the usual spectrophotometry [10,11]. Fluorescent techniques have also been implemented for DNA detection by using fluorophore-labeled oligonucleotide probes attached to GNPs by covalent chemical modification [12] or by physical adsorption [13].

Recently, a new method has been proposed for sensitive quantitative label-free DNA detection by using GNPs and RB as a fluorophore [14]. In this method, RB fluorescence is quenched by GNPs that are stabilized by single-stranded DNA (ssDNA) molecules (Fig. 1). Addition of a complementary DNA (cDNA) target results in hybridization of probes and targets in the formation of double-stranded DNA (dsDNA) duplexes, which cannot stabilize GNPs in salt environment, thus causing their aggregation. As a result, the hybridization reaction is observed as an increase in fluorescence intensity (Fig. 1). It is evident that
the principle of the fluorescence assay is similar to that of the previously reported colorimetric and fluorescence assays [10,13,15,16], as all methods utilize the differentiation between the abilities of ssDNA and dsDNA to stabilize GNPs in a salt environment. The possible physicochemical mechanisms behind this differentiation were discussed by Li and Rothberg [15,16].

It is useful to discuss shortly the surface plasmon resonance (SPR) concept underlying the DNA assay being considered. In our case, both the exciting (520 nm) and the emitted (575 nm) light wavelengths lie within the SPR band of individual 15-nm GNPs. In general, interactions of a dye molecule with the SPR particle can be enhanced or quenched [17–20]. The enhancement of fluorescence owing to the plasmonic particle is related to the local field near the particle and is closely related to the well-known electromagnetic contribution to surface-enhanced Raman scattering (SERS) [21–23]. In a first approximation, the electromagnetic enhancement of the SERS scales as $|E|^2$, thus giving rise to a giant amplification of SERS spectra at sites known as “hot spots” [24,25]. In terms of a two-staged model [20,26], the enhanced local SPR field results in an enhanced fluorescence of a dye molecule located near a plasmonic particle at the first stage. At the second stage, the fluorescence light with an increased quantum yield is radiated to its environment (radiative decay loss). On the other hand, when the gap between the particle and the dye molecule is sufficiently small, the major portion of emitted energy will dissipate by the metal particle. These phenomena are more prominent for particle clusters in which the electromagnetic “hot spots” arise near small gaps between the metal surfaces [23,24]. A very simple example is a dimer of plasmonic particles [20,27]. For obtaining maximal fluorescence, the emitting molecule should be placed at an optimal distance from the “hot spot.” Although this distance can be controlled with a nanometer accuracy [28], metallic colloids demonstrate quenching rather than enhancement in typical experiments [19]. An additional complicating factor is the strong dependence of the SPR band on particle clustering [29,30], which results in strong deviations in the absorption and scattering spectra of colloids even in the simplest case of dimers [31,32]. Specifically, aggregated colloids exhibit red-shifted and greatly broadened spectra [29,33]. Clearly, the aggregation can cause strong deviations in SPR quenching and enhancement owing to the appearance of new “hot spots” and owing to their spectral movement to red.

In addition to the enhancement and quenching phenomena, SPR colloids exhibit a more trivial yet important mechanism, which can greatly alter the measured fluorescence signals. To simplify the question, let us consider a single dye molecule located right in the center of a cuvette filled with a colloid. Owing to the SPR absorption and scattering bands, the colloid acts as a selective filter. Therefore, the incident (exciting) light will be attenuated after traveling a pass of l/2 in accordance with Bouguer’s law, $I(\lambda_{em})\exp\{-N(C_{ext})\lambda_{em}(l/2)\}$. Here, $N$ is the particle number concentration, and $C_{ext}$ is the extinction (absorption + scattering) cross-section. For an ideal measuring scheme with a zero-view detector, we will record not the fluorescence intensity $I(\lambda_{em})$ but the following quantity [34]: $I(\lambda_{em})\exp\{-N(C_{ext})\lambda_{em}(l/2)\}$. In the literature, such a selective distortion of the measured fluorescence is called the “inner field effect.” Again, it is clear that for plasmonic systems, this effect can greatly affect the measured responses in both aggregated and nonaggregated colloids.

The optical mechanisms underlying the aggregation-induced increase in fluorescence intensity may be as follows [14]. For aggregated particles, the total surface available for adsorption of RB is reduced, thus resulting in an increase in the concentration of free RB molecules and in a corresponding increase in fluorescence intensity. The second factor is known as the above discussed inner filter effect [34].

In our case (Fig. 1), the main contribution to the inner filter effect comes from the selective absorption by isolated or aggregated GNPs. In particular, for aggregated GNPs, the absorption at 520 nm decreases and the intensity of the exciting light increases correspondingly. In addition, the absorption of emitted light, traveling from the exciting point to the detector, diminishes. Eventually, the recorded fluorescence intensity of RB can be higher than that in the case of isolated GNPs.

Although these possibilities were noted by Zhang et al. [14], it remained unclear which of these possibilities (or both) constituted the physical mechanism behind the GNP-based fluorescence test. In this work, we investigate the GNP quenching and the inner filter factors in detail to show that both mechanisms are involved in the increase in overall fluorescence intensity recorded after hybridization of the probe and target ssDNA. In addition, we evaluate the detection limit for the target cDNA, which is close to that for the colorimetric and light scattering assays [11].

2. Experimental details

2.1. Reagents

All 21-mer synthetic ssDNAs, corresponding to portions of the human immunodeficiency virus type 1 (HIV-1) U5 long terminal repeat (LTR) sequence, were purchased from Syntol (Russia). Their base sequences are as follows: probe DNA sequence, 5′-ATGTGAAAAATCCTAGCATG-3′; target DNA sequence, 5′-ACTGCTAGATTTCACAT-3′; and the
noncomplementary DNA target sequence was similar to the probe DNA sequence.

To fabricate GNPs, we used the following reagents: tetrachloroauric acid, HAuCl4 (Alfa Aesar, USA); sodium citrate, Na3C6H5O7·2H2O (Sigma, USA) and Milli-Q water. To ensure hybridization conditions, we used Tris, C4H11NO3 (Helicon, Russia); HCl, and NaCl (Reachim, Russia); phosphate buffer in tablets (PB; pH 7.4), 0.01 M (Biolot, Russia); EDTA (Serva, Germany). Rhodamine B (Soyuzreaktiv, Russia) served as a fluorescent dye.

**TE** buffer (pH 7.4) consisted of 10 mM Tris–HCl and 1 mM EDTA. The hybridization buffer (HB; pH 7.4) consisted of 10 mM Tris–HCl, 1 mM EDTA, and 50 mM NaCl. The detection buffer (PBS) consisted of 10 mM PB and 0.3 M NaCl. All solutions were stored at 4 °C before use.

### 2.2. Fabrication of nanoparticles

GNPs of 15-nm diameter were fabricated by reducing HAuCl4 with sodium citrate by the method of Frens [35], with minor modifications as described by Grabar et al. [36]. Briefly, 25 mL of 38.8 mM sodium citrate was added quickly to boiled 250 mL of a 1 mM water solution of HAuCl4, resulting in a change in solution color from pale yellow to deep red. The average particle size can be tuned by varying the amount of added sodium citrate as described in Ref. [37]. Fig. 2 shows a typical TEM image of 15-nm gold particles and a corresponding size-distribution histogram. According to the TEM analysis, the average particle diameter was 15.9 ± 1.4 nm.

### 2.3. Measurements

Extinction spectra were recorded with a Specord BS-250 and a Specord S-300 VIS spectrophotometer (Analytik Jena, Germany). Fluorescent spectra were recorded with a Perkin-Elmer LS-55 (Perkin-Elmer, USA) and an Avantes AvaSpec-2048 (Avantes BV, Netherlands) spectrofluorimeters by setting the excitation wavelength at 520 nm and recording the emission intensities at wavelengths ranging from 540 to 650 nm. Throughout the study, the maximal fluorescence intensities were measured at 575 nm. The GNP average diameter was controlled with a Libra-120 transmission electron microscope (Carl Zeiss, Germany) and with a Zeta sizer Nano ZS dynamic light scattering instrument (Malvern, UK).

### 3. Results and discussion

#### 3.1. Influence of ssDNA molecules and NaCl from the hybridization buffer on RB fluorescence

Before investigation of the quenching of RB molecules by GNPs, we estimated whether the components of the reaction mixture, namely ssDNA, NaCl from the hybridization buffer, and low-molecular weight components of the GNP supernatant liquid, can influence RB fluorescence. To examine ssDNA effects, we prepared the following three solutions: a reference aqueous RB solution and two aqueous RB solutions containing 300 nM and 1 μM of probe ssDNA. For each solution, the RB concentration was constant and equal to 1.5 μM [14]. The fluorescence spectra were measured for all three samples. It was found that the maximal fluorescence intensities at 575 nm for the ssDNA-containing solutions were lower than that for the reference solution by 0.2–0.5% only (Table 1). Thus, the addition of ssDNA to the RB solution changes fluorescence intensity negligibly and does not affect the fluorescence test.

To investigate the influence of NaCl and the GNP supernatant liquid on RB fluorescence, we prepared an aqueous RB solution, RB in an NaCl solution (final concentration of the detection buffer, 86 mM), and RB dissolved in the supernatant liquid obtained after centrifugation of GNPs. The optimal concentration of NaCl (86 mM) for the maximal fluorescence response was found by Zhang et al. [14] and was confirmed in our experiments.

As shown in Fig. 3, the fluorescence intensity of RB in the salt solution was somewhat higher than the intensity for the aqueous RB solution. This result was reproduced in several independent experiments, though the maximal intensity was different from run to run. Although the precise origin of such enhanced fluorescence in 86 mM NaCl solution is currently unknown, we speculate that it can be related to dissociation of RB aggregates (dimers, trimers, etc.) in a salt environment. In contrast to the enhanced fluorescence in a salt environment, we observed quite negligible differences between spectra 1 and 3. This strongly suggests that the effect of the low-molecular

<table>
<thead>
<tr>
<th>Sample</th>
<th>ssDNA concentration (μM)</th>
<th>RB concentration (μM)</th>
<th>Fl. intensity normalized to reference sample 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>1.5</td>
<td>0.98</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.5</td>
<td>0.95</td>
</tr>
</tbody>
</table>

**Table 1**

Effect of ssDNA on RB fluorescence.

![Fig. 2. TEM image of GNPs obtained with a Libra-120 (Carl Zeiss) electron microscope. The inset shows a number–size distribution histogram. Average particle diameter, 15.9 ± 1.4 nm.](image-url)
weight components of the GNP supernatant liquid (citrate ions, etc.) on RB fluorescence was negligible.

It should be noted that curve 2 in Fig. 3 demonstrates the maximum observed difference between the aqueous and the salt-containing solutions of RB. Typically, the maximal difference was in the range of 2–5%, whereas the difference between the maxima of spectra 1 and 2 in Fig. 3 was about 15% at 575 nm. Thus, we can conclude that the probe ssDNA, NaCl salt from the hybridization buffer, and the low-molecular-weight components of GNP solutions did not significantly affect the RB fluorescence spectra. However, the recorded variations of fluorescence in a salt environment could reduce the assay sensitivity and increase the low detection limit (see below).

3.2. Effect of the GNP concentration on the fluorescence spectra of RB

The effect of GNPs on the fluorescence properties of RB has been studied by several authors [38–40]. The fluorescence intensity was shown to be strongly dependent on the GNP size [38], concentration [40], and charge [41]. As RB is a cationic dye [14,41], it can easily be adsorbed on negatively charged citrate GNPs. Depending on the GNP–RB composition, one can observe fluorescence enhancement by GNPs [38–41] or quenching of fluorescent molecules located near metal nanoparticles [34,42].

The fluorescence quenching by GNPs is caused by an increased nonradiative rate and a drastic decrease in the dye's radiative rate [40,42–44]. By contrast to the fluorescence enhancement experiments with GNP films [38–41], fluorescence quenching is usually observed for diluted colloids [19]. In particular, the experimental data of Zhang et al. [14] show that at a GNP concentration of 6.9 nM and an RB concentration of 1.4 μM, strong quenching of RB by GNPs was observed at a moderate ratio of about 200 RB molecules per one 15-nm gold particle. However, the fluorescence spectra of Zhang et al. [14] were not corrected to the inner filter effect, which can obscure the net GNP quenching. Below we provide a set of experimental data aimed at elucidating the GNP concentration effect on fluorescence quenching with the inner filter correction being taken into account.

Fig. 4a shows relative fluorescence intensity as a function of the GNP concentration. Experimental curve 1 was obtained at 575 nm and a constant RB concentration of 1.5 μM with a 1-cm cuvette and an AvaSpec-2048 instrument. The GNP concentrations \( N = 3.8 \times 10^{12} A_{520} \text{ (particles/mL)} \) were found from the optical densities \( A_{520} \) of GNP solutions at 520 nm and from the integral extinction cross-section calculated by Mie theory [45].

Curve 1 in Fig. 4a represents uncorrected experimental measurements, whereas curve 2 was corrected to the inner filter effect according to the following approximate equation [34]:

\[
I_{\text{corr}} = 10^{(A_{520} + A_{575})/2} I_{\text{exp}},
\]

(1)

where \( A_{520} \) and \( A_{575} \) are the optical densities at the excitation and emission wavelengths. Eq. (1) accounts for the absorption of exciting light, which travels to the cuvette's center, and emitted light, which travels from the cuvette's center to the detector. In other words, both geometrical passes within the cuvette are assumed to equal \( l/2 \) (see the insets in Fig. 4). Actually, the inner filter
effect is manifested as the difference between the measured and the corrected curves. The course of corrected curve 2 can be explained by an increased amount of RB molecules adsorbed on GNPs and their quenching.

At high GNP concentrations (e.g., 6.9 nM, used by Zhang et al. [14]), the difference between the measured and the corrected intensities can be of about one order. However, the actual inner filter correction depends strongly on particular instrument geometry. For example, Eq. (1) only holds for a thin parallel or focused exciting beam and for a detector with a zero viewing angle. As the AvaSpec-2048 instrument does not meet these requirements, we also performed similar measurements with a Perkin-Elmer LS-55 spectrofluorimeter (Fig. 4b). It is evident that this instrument was more suitable for our systems containing GNPs and RB with overlapping absorption bands. In any case, the inner filter effect was reduced significantly as compared with that shown in panel (a). On the other hand, to make the inner filter effect more evident, we used the AvaSpec-2048 instrument in preference to the Perkin-Elmer LS-55 spectrofluorimeter.

The next experiments were done with the same samples as in Fig. 4a but now with the addition of NaCl to initiate aggregation of GNPs (Fig. 5). In these experiments, NaCl was added to an aqueous solution containing 1.5 μM RB and GNPs; the final NaCl concentration was equal to that used for hybridization conditions, 86 mM (Fig. 5b). In a sense, such colloids imitate the spectral properties of DNA-containing systems formed during the hybridization process [11,14]. Actually, the left panel in Fig. 5a reproduces the plots in Fig. 4a, measured with the AvaSpec-2048 instrument. The addition of the salt led to a drastic change in the colloid spectra and, accordingly, to significant differences between the measured intensities for nonaggregated and aggregated systems (compare curves 1 in Fig. 5a and b, respectively). This means that the measured fluorescence intensity can be greatly distorted owing to GNP aggregation, as compared with a nonaggregated colloid. However, it would be erroneous to explain these differences between curves 1 by the net reduction in quenching. Indeed, only the difference between the corrected intensities (i.e., between curves 2 in Fig. 5a and b) can be attributed to a significant reduction in fluorescence quenching by aggregated GNPs. Experimentally, this effect was observed as enhanced fluorescence after the salt addition. Similar results were obtained with the Perkin-Elmer LS-55 instrument (data not shown), although the absolute variations in the intensity of the corrected and noncorrected curves vs the GNP concentration differ from those shown in Fig. 5.

3.3. Correlation between the adsorption of RB molecules on GNPs and the fluorescence intensity

Solutions with a constant final RB concentration (1.5 μM) were incubated with GNP colloids of concentrations ranging from 0 to 5.2 × 10^{12} particles/mL for 30 min. Then, the mixtures were centrifuged at 10,000g for 30 min and the fluorescence intensities of the supernatant liquids I_s were measured at 575 nm. Fig. 6 shows the experimental scheme used to measure the RB adsorption curves in terms of the ratios I_s/I_0 and (I_s−I_0)/I_0, where I_0 is the fluorescence intensity of a reference aqueous RB solution at 575 nm. CF stands for centrifugation of samples after 30-min incubation. In a sense, the (I_s−I_0)/I_0 plots in Fig. 6 can be considered to be an adsorption isotherm for RB molecules adsorbing on GNPs. Indeed, the difference between I_s and I_0 correlates with the amount of RB molecules adsorbed on GNPs; therefore, the ratio (I_s−I_0)/I_0 is proportional to the fraction of adsorbed RB molecules. With an increase in the amount of added GNPs, the fraction of adsorbed molecules should increase from 0 to 1, provided that the adsorption is not reversible. Owing to the fluorescence quenching of the adsorbed RB, the ratio I_s/I_0 should decrease from 1 (the initial RB solution without GNPs) to 0 (complete adsorption and quenching of RB on GNPs).

It follows from Fig. 6 that RB molecules are practically absent from the supernatant liquids obtained for colloids with the maximal GNP concentration. In addition, Fig. 4 shows negligible fluorescence for the RB+GNPs systems at the same maximal GNP concentrations. Therefore, these experiments give unambiguous evidence for a direct correlation between complete disappearance of fluorescence and complete adsorption of all RB molecules on the GNPs, as it follows from our measurements of the supernatant liquids. Consequently, at the maximal GNP concentration (about

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**Fig. 5.** Fluorescence intensity as a function of the GNP concentration, as measured for NaCl concentrations of 0 (a) and 86 mM (b). For details, see the text.
fluorescence was completely quenched owing to the adsorption of RB on metal particles rather than owing to the inner filter effect. It is obvious that this quenching mechanism plays a crucial role in the fluorescence assay based on DNA induced aggregation of GNPs in solutions containing RB molecules.

### 3.4. Quenching of RB fluorescence by GNP+ssDNA complexes

In Section 3.1, we have shown that the dissolved ssDNA molecules did not affect the RB fluorescence intensity. However, the adsorption of RB on bare GNPs resulted in quenching of RB fluorescence (Sections 3.2 and 3.3). In this section, we examine whether the ssDNA being adsorbed on a GNP can interact with RB and can influence RB quenching. In other words, we here examine whether RB molecules can be quenched by GNP+ssDNA complexes in the same manner as they can be quenched by bare GNPs.

Table 2 lists the optical densities and the relative fluorescence intensities for six samples consisting of RB molecules, bare GNPs, or GNPs preincubated with ssDNA. For details, see the text.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>(N \times 10^{12}) m(^{-1})</th>
<th>(A_{520})</th>
<th>(\delta A) (%)</th>
<th>(I/I_0) (a.u.)</th>
<th>(\delta I) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GNP+RB</td>
<td>2.8</td>
<td>0.79</td>
<td>0</td>
<td>0.42</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>GNP+300 nM ssDNA+RB</td>
<td>2.8</td>
<td>0.83</td>
<td>5.1</td>
<td>0.35</td>
<td>−17</td>
</tr>
<tr>
<td>3</td>
<td>GNP+1 μM ssDNA+RB</td>
<td>2.8</td>
<td>0.85</td>
<td>7.6</td>
<td>0.38</td>
<td>−9.5</td>
</tr>
<tr>
<td>4</td>
<td>GNP+RB</td>
<td>5.1</td>
<td>1.40</td>
<td>0</td>
<td>0.033</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>GNP+300 nM ssDNA+RB</td>
<td>5.1</td>
<td>1.43</td>
<td>2.1</td>
<td>0.035</td>
<td>+6.3</td>
</tr>
<tr>
<td>6</td>
<td>GNP+1 μM ssDNA+RB</td>
<td>5.1</td>
<td>1.39</td>
<td>−0.7</td>
<td>0.027</td>
<td>−21</td>
</tr>
</tbody>
</table>

It follows from the data of Table 2 that the optical densities of the GNP+ssDNA complexes after the addition of RB molecules do not differ significantly from the optical densities of the reference solutions. The fluorescence intensity variations were higher than the variations in optical densities. The initial fluorescence intensity of RB solutions without bare GNPs or GNP+ssDNA complexes was equal to 57 a.u.—about 30 times higher than that for fluorescence intensities of the last three samples. Therefore, we conclude that almost complete quenching of RB had occurred for all the samples.

From these experiments, we conclude that the interaction between GNPs and ssDNA does not change significantly the RB quenching and should not interfere with the DNA fluorescence test.

### 3.5. Enhancement of RB fluorescence by GNPs at low GNP concentrations

According to the model proposed by Gersten and Nitzan [46], there are two mechanisms of fluorescence enhancement related to the enhancement of the local field at the excitation wavelength and to the change in the partitioning of the excitation energy. In the case of GNPs, the field enhancement is induced by excited localized surface plasmons, whereas the second mechanism comes from modification of decay channels by the plasmonic nanoparticle [39,42]. It should be emphasized that the plasmonic enhancement of fluorescence has been observed for specific experimental conditions [38–41] at very high RB/GNP molar ratio and for negatively charged particles only [41].

Here, we describe our observation of GNP-enhanced RB fluorescence in the case of low GNP concentrations and a low RB/GNP molar ratio. The enhanced fluorescence was recorded at a constant RB concentration of 1.5 μM and for GNP concentrations below \(10^{12}\) particles/mL with a Perkin-Elmer LS-55 instrument equipped with a 3-mm glass cuvette. The measurements were made at 2 and 24 h after mixing of RB and GNP solutions. In general, the measured intensities were similar for both experimental conditions.
set of observations, so the spectra were averaged over a 2- and a 24-h incubation time.

Fig. 7 shows the averaged fluorescence spectra of aqueous mixtures of RB and GNPs and the dependence of the intensity maxima $I_{575}$ on the GNP concentration. All the measurements were done in a 3-mm cuvette with a Perkin-Elmer LS-55 instrument. In general, the concentration dependence in panel (b) resembles that reported by Levchenko et al. [41] for 13.6-nm GNP size, which is close to the size of our GNPs. However, their RB (50 μM)/GNP (4 μM) = 12.5 M ratio was almost 250 times higher than our ratio RB (1.5 μM)/GNP (30 μM) = 0.05. Zhu et al. [40] also reported a concentration-dependent fluorescence enhancement by GNPs with a maximum near the GNP concentration of 14 μM. Again, their RB/GNP molar ratio was about 5, almost two orders higher than our RB/GNP ratio of 0.05. Moreover, Zhu et al. [40] used 40-nm GNPs; therefore, we had to compare the numbers of molecules per unit GNP surface rather than the molar ratios. As the specific surface of a colloid scales inversely with the particle size at a constant molar or mass/volume concentration, the number of RB molecules per unit GNP surface in our case was almost three orders lower. In their explanation of the GNP-induced fluorescence enhancement, Zhu et al. [40] used a model for an RB-capped gold nanosphere to calculate the local electrostatic filed enhancement. As any optically dense dielectric shell shifts the plasmon resonance to longer wavelengths [47], the local field factor first increases and then decreases.

The exact reasons for the recorded nonmonotonic dependence in Fig. 7b are currently unknown, because the model [40] hardly holds in our case. The electromagnetic mechanism of enhancement seems doubtful, because we observed fluorescence quenching for an increasing GNP concentration, even after the inner filter correction. Another possible explanation is an inverted inner filter effect, caused by a red-shifted GNP extinction spectrum, which, in turn, results in an increased beam intensity at the excitation wavelength. In any case, the small size of our particles is not optimal for observation of a significant enhancement of fluorescence. For example, Nakamura and Hayashi [38] found a size-dependent enhancement with an optimum near 150 nm. As the high RB/GNP molar ratio is not suitable for DNA detection based on a GNP-induced quenching mechanism, we did not investigate GNP-enhanced fluorescence in detail. This important question is beyond the scope of this work and deserves further study.

3.6. DNA detection assay based on fluorescence quenching of Rhodamine B by GNPs

Zhang et al. [14] performed a thorough optimization of the DNA detection assay with regard to NaCl, probe DNA, and target cDNA conditions. Therefore, our experimental conditions for DNA detection and the assay protocol were the same as those used in [14]. However, in contrast to the data of Zhang et al. [14], we observed rather worse reproducibility of assay results and, accordingly, our detection limit was significantly higher than that reported in [14].

The top row in Fig. 8 shows the colors of tubes with GNPs mixed with solutions of ssDNA (tube 8; noncomplementary target, 300 nM), with dsDNA after hybridization of the probe and target DNA (tubes 1–7; target cDNA concentrations, 300, 30, 3, 0.3, 0.03, 0.003, and 0.0003 nM), and finally with the detection buffer and RB solution (tube 9). Other reaction conditions are as follows: GNP concentration, $5.3 \times 10^{12}$ ml$^{-1}$; concentration of probe ssDNA in hybridization buffer, 300 nM; and RB concentration, 1.5 μM.

The bottom row shows the same samples irradiated with a UV flatbed fluorescence detector. For the left top sample with the highest concentration of the target cDNA (300 nM), the solution color changed from red to purple, indicating the aggregation of GNPs. In the bottom row, this left tube fluoresced most intensely, although this difference from other fluorescent tubes is quite small for the naked eye. Therefore, because of the small changes in the color of the top-row colloids and in the fluorescent intensities of the bottom-row tubes, we cannot expect that the method will be highly sensitive below nanomolar cDNA
We estimate a realistic low detection limit (LDL) to be in the intensity. Taking into account the typical experimental errors, to 0.3 nM led to quite a small, 10% increase in the fluorescence and for the minimal cDNA concentration (0.3 pM) is not between the intensities for the blank sample ([cDNA] logarithmic). Because of random variations, the difference in the fluorescence intensity (note that the abscissa scale is nonlinear) between the cDNA concentration and [48] and 10 pM LDL for the dynamic light scattering assay previously reported 100 pM LDL for the colorimetric assay [14]; however, it disagrees with the result of Zhang et al. [10,11].

4. Conclusions

We have studied the optical mechanisms underlying the DNA detection assay based on fluorescence quenching of the fluorescent dye Rhodamine B by GNPs. The assay principle is based on the different ability of single- and double-stranded DNA to stabilize GNPs in a salt environment. In the first set of experiments, we have shown that the probe ssDNA and the low-molecular components of GNP solutions do not affect the RB fluorescence spectra, although we have observed notable variations in the spectra for different NaCl concentrations, in agreement with previous observations [14].

In Section 3.3, we have examined the fluorescence of GNP+RB supernatant liquids and we have provided an unambiguous proof for a direct correlation between complete fluorescence quenching and complete adsorption of all RB molecules on GNPs. Our main result is that both the inner filter effect and the quenching of the dye owing to its adsorption on GNPs contribute to the DNA detection assay based on measurements of fluorescence in mixtures of GNPs, ssDNA, dsDNA, and the RB dye. Finally, according to our estimates, the LDL of the fluorescent DNA assay lies in the range 30–100 pM, which is close to the LDLS reported previously for DNA assays based on extinction spectra and on static and dynamic light scattering measurements.

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