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Nanoparticle Aggregate-Based Fluorescence Enhancement for Highly Sensitive and Reproducible Detection of DNA

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With the development of new synthesis routes and deeper understanding of their properties, noble metal nanoparticles have been extensively studied for use in different biosensing applications.[1] Although non-optical characteristics of the nanoparticles, such as catalytic[2] or electrical properties,[3] have been applied for sensing, most of the published research on the use of nanoparticles for biosensing is related to their optical properties. Two main types of optical properties of nanoparticles are utilized in biosensing applications. The first is the strong interaction of the metal nanoparticle with light, based on the plasmonic nature of the particle, and its sensitivity to the environment. Using these properties, different types of biosensing assays were developed, for example, the nanoparticle was used as a tag in a surface-bound sandwich assay,[4] the plasmon–plasmon interaction between close particles was used to report the existence of biomolecule-based crosslinking,[5] and the change in the position of the plasmon resonance of the nanoparticle was used to sense a biorecognition event.[6] The second group of optical properties that are used in sensing applications is based on the modification of optical properties of molecules in the vicinity of nanoparticles due to the high local fields that can be generated. Several types of spectroscopies can be enhanced by the local field,[7] giving rise to diverse phenomena such as surface-enhanced Raman scattering (SERS), surface-enhanced fluorescence (SEF), and surface-enhanced infra-red absorbance (SEIRA). SERS has been demonstrated as a useful technique for multiplexed DNA detection[8] and protein detection.[9] Recently, there has been a growing interest in the applications of SEF for biosensing applications.[10] One of the main benefits of using SEF rather than SERS in diagnostic assays is the availability of fluorescence microscopes and fluorescence plate readers in most biochemistry labs, whereas Raman-based detection systems, are still rare in such settings. While the majority of the literature on biosensing applications of SEF involves nano-textured surfaces,[11] only very few reports on enhancement in solution have been published.[12] As most surfaces that provide high fluorescence enhancement involve expensive lithographic processes, solution-based SEF that involves unmodified nanoparticles holds promise for low-cost assays.

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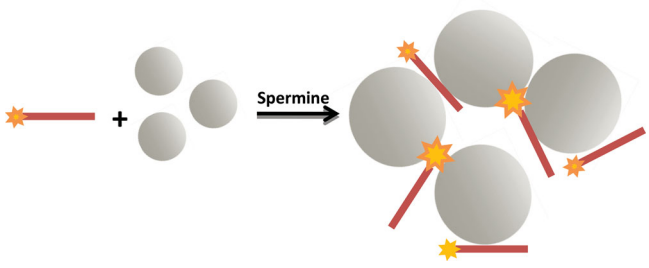
We have recently shown that fluorescence enhancement can be observed from dye-labeled DNA when mixed with silver nanoparticles in the presence of the polycation spermine[13] (see **Scheme 1**). Although this behavior was shown for a wide variety of dyes, dyes in the red edge of the spectrum, such as Atto-655, have shown the highest enhancement (more than 150× enhancement in fluorescence intensity).[12a] Here, we show that this cheap and simple method can be used as an amplification step to detect and quantify DNA at sub picomolar concentrations without any enzymatic enhancement.

As a first step to demonstrate the applicability of silver nanoparticle aggregation-based SEF for DNA detection, we have looked at the effect of SEF amplification on the detection limit of dye-labeled DNA using a fluorescence microplate reader. To make both measurements comparable, we have used the same instrument and the same gain setting for both measurements. As can be seen in **Figure 1**, more than two orders of magnitude enhancement in dye fluorescence is achieved over the entire measured concentration range (from 600×10^{-12} M down to less than 0.1×10^{-12} M).

This also translated into a reduction of the detectable DNA concentration by more than two orders of magnitude, resulting in a level of detection (LOD) below 50×10^{-15} M. Another important property of fluorescence enhancement using nanoparticle aggregation is that it has a large linear range of more than three orders of magnitude. Interestingly, the process of aggregation of particles does not introduce a higher variance in the measurement data. In all measurements shown in Figure 1, the standard deviation was smaller than the marker size and therefore does not appear on the graph. For all measurements in the linear region, both without SEF and with SEF, the relative standard deviation was below 4%. An additional useful property of SEF-based amplification is that it does not add any background. Other amplification methods, such as those based on enzymes that produce a fluorogenic product, non-specific binding of materials involved in the amplification step will increase the background for non-target sample. However, in the



Scheme 1. Co-aggregation of dye-labeled DNA with silver nanoparticles.

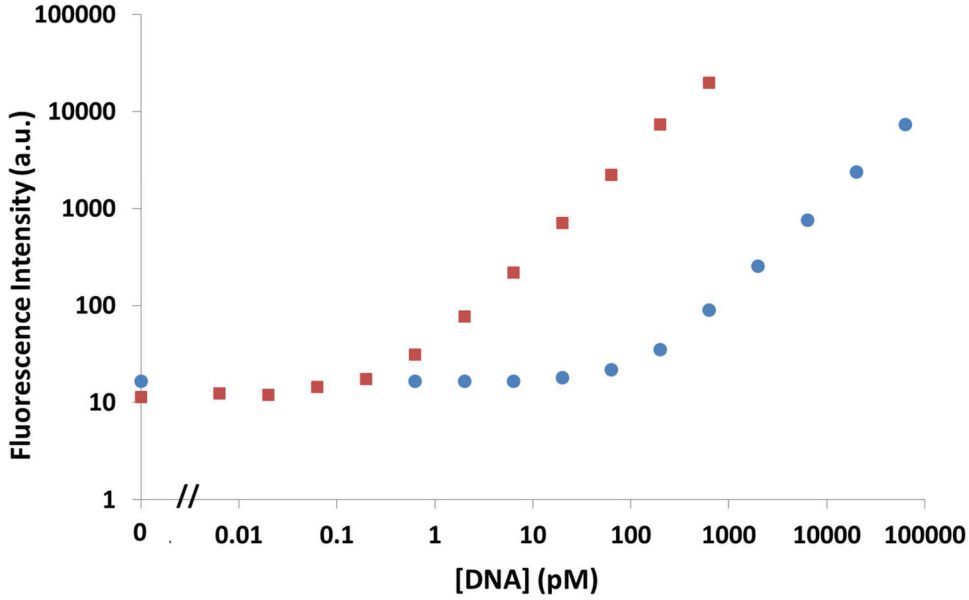


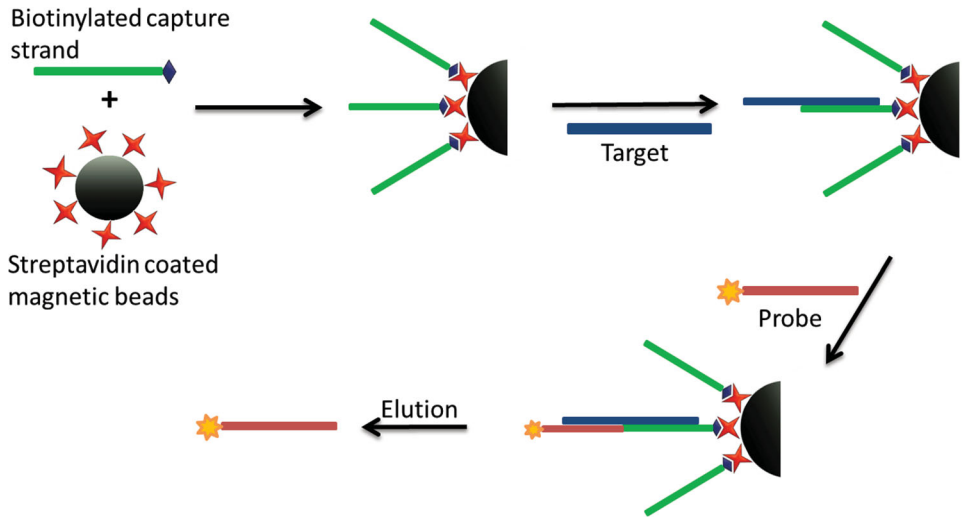
Figure 1. Fluorescence intensity as a function of probe DNA concentration for direct detection (circles) and under SEF conditions (squares).

case of SEF, since enhancement can only occur from existing fluorophores, there is no additional background added.

When designing a fluorescence-based assay to detect DNA in its natural form (not labeled with a fluorescent dye), one requires a method that can correlate the concentration of the non-labeled target DNA with a concentration of a labeled probe DNA. For this, we designed a magnetic-bead-based sandwich hybridization assay, as depicted in **Scheme 2**. Biotin-tagged capture oligonucleotides were immobilized on streptavidin-coated magnetic beads to create capture probe-modified magnetic beads. The modified beads could then specifically hybridize to one part of the target stand. A dye-labeled probe DNA was designed to hybridize to a second part of the target strand. After washing away the excess probe, one can relate the number of targets that were captured by the beads, to the number of probes that were attached to the beads. However, steric hindrance will prevent effective SEF by silver particles when the probe DNA is still bound to the magnetic beads, as the DNA would not be positioned at the optimal areas (the hot spots). Hence, an additional elution step was added. We then split the eluent in two parts, in order to achieve a fair comparison between direct detection of the labeled probe and detection using SEF-based signal amplification.

In addition for optimization procedures which are usually done in magnetic-bead-based assays (for example, hybridization time and magnetic bead concentration—see Figure S3 and Figure S4, Supporting Information), we also had to make sure that the optimized elution buffer does not interfere with the SEF enhancement step that follows.

For the elution conditions optimization, we choose to use a two-part hybridization assay, where the target was dye labeled, instead of using the full three-part hybridization assay, in order



Scheme 2. Magnetic-bead-based sandwich hybridization assay for the detection of DNA.

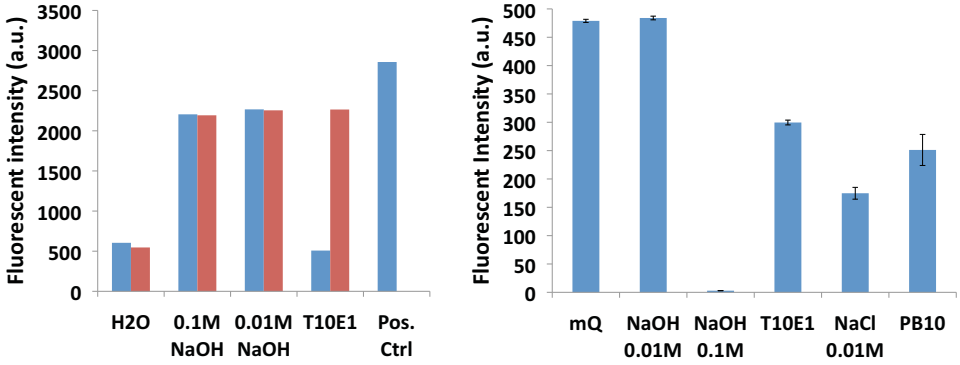


Figure 2. A) fluorescence intensity of eluted FAM-labeled target using different elution buffers at RT (blue) and 95 °C (red). B) Fluorescence intensity of the probe DNA A655-tag diluted in different buffers to a final concentration of 100×10^{-12} M. The fluorescence was measured under SEF conditions. T10E1 is 10×10^{-3} M Tris buffer pH 7.5 with 1×10^{-3} M of EDTA. PB10 is 10×10^{-3} M phosphate buffer, pH 6.6.

to minimize the variance between experiments. To determine the elution efficiency, dye-labeled targets were captured by capture-probe immobilized magnetic beads, washed, and then put in the elution solution for 10 min. After magnetic separation, the fluorescence of the supernatant was compared with the fluorescence of the original target concentration in the same elution buffer. As can be seen in **Figure 2**A, for near neutral pH conditions, effective elution was possible only at a high temperature. However, at basic pH, in the presence of NaOH, efficient elution was observed both at room temperature and at elevated temperatures. Once we established the conditions for most efficient elution, we looked at the effect of the eluent on the SEF signal. Our previous experience showed that most buffers (such as phosphate buffer) or even the addition of salt (NaCl) at low levels (10×10^{-3} M) caused a significant drop in the enhanced fluorescence intensity. As can be seen in Figure 2B, when the dye-labeled probe is diluted in 10×10^{-3} M NaOH instead of in water, no significant difference in fluorescence is observed. This is in contrast to the 100×10^{-3} M NaOH or 10×10^{-3} M of Tris (or phosphate) buffer, where a pronounced reduction in fluorescence under SEF conditions is observed. We attribute the reduced effectiveness in the enhancement of the fluorescence when using a high concentration of NaOH to the partial deprotonation of spermine at very high pH, resulting in an ineffective charge neutralization of the DNA. At the lower NaOH concentration, the buffering effect of the other components (which contribute 80% of the total volume) keeps the conditions similar to those which occur when the sample is in water.

Finally, we have compared the detection of unlabeled target DNA using the magnetic bead hybridization assay with and without the fluorescence enhancement step of the silver nanoparticle aggregation. **Figure 3** shows the calibration curve for this assay. The detection limit for this assay is 200×10^{-12} M without SEF, and 500×10^{-15} M with SEF. Again, we have obtained very low standard deviations (in four repeats), which were less than 9% for the assay without SEF and less than 10% for the assay with SEF, over the whole detectable

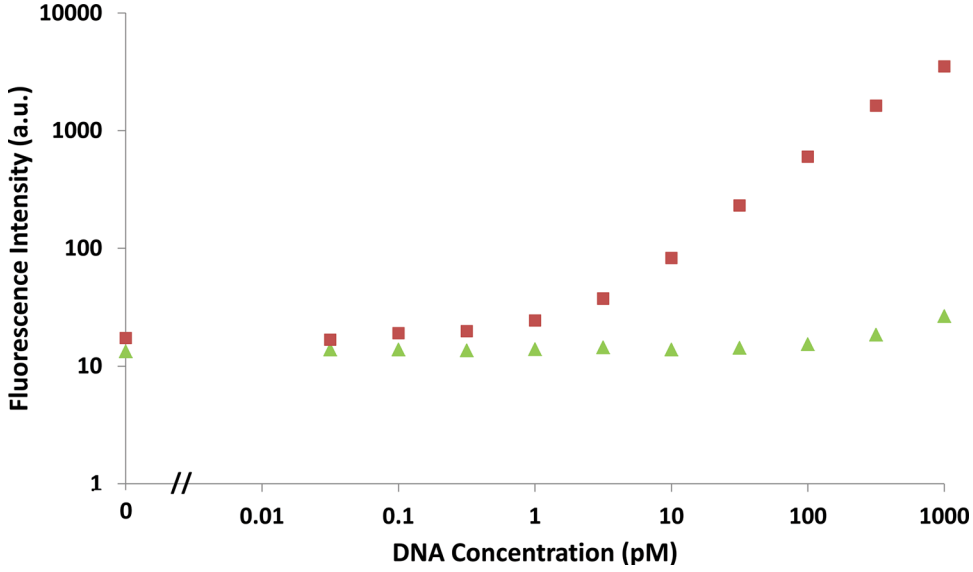


Figure 3. Fluorescence intensity as a function of target DNA concentration when using the magnetic-bead-based assay for direct detection (triangles) and under SEF conditions (squares).

Table 1. Sequences of the oligonucleotides used in this study.

Name of DNA	Sequence
Biotin-capture	5′- ACG CCT TCT TGT TGG AAA-biotin-3′
Target	5′- CCA ACA AGA AGG CGT AAG ACG TTA TCC ACC-3′
A655-tag	5′- Atto655-TGG AAG TCA GAT GGT GGA TAA CGT CTT-3′
Thiol-capture	5′- thiol-AAA AAA AAA GGT GGA TAA CGT CTT-3′
Target-FAM	5′- CCA ACA AGA AGG CGT–(U-FAM)-AAG ACG TTA TCC ACC-3′
Non-target	5′- TGG AAG TTA GAT TGG GAT CAT AGC GTC AT-3′

concentration range. Additional control experiments done with a high concentration (100×10^{-6} M) of non-target control using a similar sized DNA sequence showed no statistical difference between the no-target (blank) control and the non-specific target control. (see Figure S1, Supporting Information).

An important factor that we are frequently questioned about is the effect of DNA probe sequence on the SEF-based fluorescence enhancement. We previously reported on the use of[12a] a literature-based sequence designed for Taqman-based detection of the MecA gene of *Staphylococcus aureus*.[14] The choice for this sequence was based on the availability in our lab, and not because it was optimized for SEF. A different probe sequence (from the same paper) used for the detection of the fem-A gene was giving higher enhanced fluorescence in our initial tests.[13] A totally different type of DNA sequence that we are currently looking at for extending the application of SEF for protein detection, the anti-VEGF aptamer V7T1,[15] also shows high fluorescence under SEF conditions (see Figure S2, Supporting Information). However, this does not mean that all sequences lead to high fluorescence enhancement. We have noticed, that short DNA sequences (below 20–22 nucleotides) usually do not give high enhancement (see Supporting Information). Additionally, artificial DNA sequences we designed, that had only T bases, or T bases with a few G bases near the dye did not give an enhanced fluorescence signal as high as those sequences that had at least two of the nucleotides A or C in the first six bases (see Supporting Information). We are currently investigating possible explanations for this phenomenon.

In conclusion, we have shown that by using SEF from dye-labeled DNA co-aggregated with silver nanoparticles, two orders of magnitude reduction in the detection limit could be achieved. By combining the enhanced fluorescence effect with a magnetic-bead-based sandwich hybridization assay, sub picomolar concentrations of unlabeled DNA could be detected and quantified. This method of fluorescence enhancement is very reproducible, leading to a barely detectable increase in the coefficient of variance for repeat experiments. We are currently pursuing the extension of this concept to protein detection.

Experimental Section

Materials and Reagents: Synthetic oligonucleotides were purchased from IBA-GmbH (Göttingen, Germany), see **Table 1** for sequences. The chemicals spermine (≥99.5% purity), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, tris(2-carboxyethyl)phosphine (TCEP), and silver nitrate (99.9999% purity) were purchased from Sigma–Aldrich. Streptavidin-coated Bio-Estapor magnetic microspheres, 1 µm diameter, were purchased from Merck Chimie SAS (Fontenay-sous-Bois, France). Maleimide-PEG2-Biotin was purchased from Thermo Scientific.

Nanoparticles Synthesis: EDTA-capped silver nanoparticles were synthesized as described previously[16] with small modifications. Briefly, 500 mL 0.16×10^{-3} M EDTA-acid solution containing 4×10^{-3} M NaOH was heated to boiling. Under stirring, four aliquots of 1.25 mL 26×10^{-3} M $AgNO_3$ were added and the solution was held at boiling for 20 min.

DNA Immobilization on Magnetic Microspheres: 1 nmol "thiol-capture" DNA was reduced by 2 nmol TCEP and conjugated with 10 nmol maleimide-PEG2-Biotin at room temperature (RT) during a 2-h incubation to form biotin-labeled capture DNA. The biotin-labeled "thiol-capture" DNA was purified by gel filtration over an illustra NAP5-column (GE Healthcare). 500 µg streptavidin-coated magnetic microspheres were washed three times with wash-bind-hybridization buffer (WBH-buffer: 20×10^{-3} M Tris-HCl, 1×10^{-3} M EDTA and 0.5×10^{-3} M NaCl, pH 7.5). Magnetic microspheres were collected with a DynaMag spin magnet (Life Technologies). The spheres were resuspended in 100 µL of buffer containing 40×10^{-3} M Tris-HCl, 2×10^{-3} M EDTA, and 1 M NaCl. An equal volume of 0.6×10^{-6} M biotin-labeled "thiol-capture" DNA was added to the streptavidin-coated spheres and incubated at RT for 10 min under constant rotation. Unbound DNA was removed by three times washing with WBH-buffer and the beads were resuspended in 100 µL WBH-buffer. These beads were used for the two-part hybridization assay with labeled target (target-FAM).

For the three-part hybridization assay, the same protocol for the modification of the beads was used, expect that the DNA used was 0.5×10^{-6} M "biotin-capture" DNA.

Fluorescence Measurement: Fluorescence was measured on a Tecan Infinite M200 PRO microplate reader with an excitation and emission wavelength as indicated in the assays.

Detection of Normal and Enhanced Fluorescence from Dye-Labeled DNA: A concentration series from 6.3×10^{-15} M to 200×10^{-6} M of the dye-labeled DNA "A655-tag" was made in mQ-water. To measure the normal fluorescent signal of this series, 40 µL "A655-tag" was diluted in 50×10^{-3} M phosphate buffer pH 6.6 to a final volume of 200 µL and fluorescence was measured on the plate reader with excitation/emission settings of 655/690 nm. To measure the silver-enhanced fluorescence signal, 40 µL of "A655-tag" was mixed with 40 µL of 500×10^{-6} M spermine in 10×10^{-3} M Tris-Tween 0.00005%. The DNA-spermine mix was thereafter mixed with 120 µL Ag-nanoparticles buffered solution. The Ag-nanoparticles buffered solution was made by mixing the as-synthesized EDTA silver nanoparticles at a ratio of 2:3 with 10×10^{-3} M phosphate buffer pH 6.6, and left after mixing at least 1 h prior to the measurement. The final volume was 200 µL for both normal and SEF measurements. The fluorescence intensity was measured for 100 s at 10 s intervals directly after mixing. The mean fluorescence intensity of the 10 measurements is shown in the graph. Values are means of three replicates.

Effect of Different Buffers on Normal and Enhanced Fluorescence of Dye-Labeled DNA: Dilutions of 100×10^{-12} M "A655-tag" DNA were made in 10×10^{-3} M NaOH, 100×10^{-3} M NaOH, buffer of 10×10^{-3} M Tris, and 1×10^{-3} M EDTA, 10×10^{-3} M NaCl, 10×10^{-3} M phosphate buffer pH 6.6, and 10×10^{-3} M NaOH with 0.001% Tween. Measurement of SEF and plain fluorescence was done as described in Section 2.5.

Optimization of the Elution Step for the Probe DNA: To optimize the elution, the labeled target DNA "target-FAM" is directly hybridized to DNA-capture-beads in a two-part hybridization assay and eluted in different ways. 2.5×10^{-6} M "target-FAM" was hybridized to 10 µg capture-DNA immobilized microspheres in 1 × WBH-0.05% Tween 20 buffer at RT for 30 min. Unbound "target-FAM" was removed by washing twice with 0.1 × WBH-0.005% Tween 20. The elution of "target-FAM" was performed at RT and at 95 °C in mQ water, NaOH 100×10^{-3} M, NaOH 10×10^{-3} M, Tris 10×10^{-3} M, EDTA 1×10^{-3} M, and Tris 1×10^{-3} M EDTA 0.1×10^{-3} M for 5 min. After magnetic bead separation, 100 µL 200×10^{-3} M carbonate buffer pH 9.5 was added to 100 µL supernatant and fluorescence was measured on the plate reader with excitation/emission settings of 488/522 nm.

DNA Hybridization Assay with Magnetic Microspheres: In each hybridization reaction, 2 µL of the 5 mg mL^{-1} solution of capture DNA-coated magnetic spheres was used. Prior to the reaction, the spheres were washed four times in WBH-buffer with 0.05% Tween 20 to remove the storage buffer and possibly released capture DNA, and resuspended in WBH-Tween20 0.05% buffer. The target and the dye-labeled signaling DNA probe "A655-tag" were heated for 5 min at 70 °C and cooled on ice to open up any secondary structure. A dilution series of 6.3×10^{-15} M to 200×10^{-12} M "target" DNA was incubated with 2.5×10^{-6} M of the probe "A655-tag" and the "biotin-capture" immobilized spheres in 100 µL WBH-Tween20 0.05% buffer in low profile PCR strips (Sarstedt). Incubation took place for 2 h at RT under rotation to hybridize target DNA on immobilized spheres and DNA probe on target DNA. A 96-plate magnet DynaMag-96 Side (Life Technologies) was used to collect the magnetic microspheres. Excess of probe DNA was removed by three times washing with low salt buffer 0.1 × WBH-0.005% Tween. After removing all traces of wash buffer, the probe DNA was released by incubation with 100 µL 10×10^{-3} M NaOH for 10 min at RT. 40 µL of the supernatant was mixed with 160 µL phosphate buffer 62×10^{-3} M for the normal fluorescence measurement. Another 40 µL supernatant was mixed with 40 µL 500×10^{-6} M spermine in 10×10^{-3} M Tris-Tween 0.00005% and 120 µL 2:3 diluted silver nanoparticles in 10×10^{-3} M phosphate buffer. The fluorescence intensity was measured for 100 s at 10 s intervals directly after mixing. The mean fluorescence intensity of the 10 consecutive measurements was used in the graphs appearing in the paper. Excitation/emission of dye fluorescence was measured at 655/690 nm. The assay was performed in four replicates.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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