

NANOBIOTRANSDUCER FOR DETECTING FLAVESCENCE DORÉE PHYTOPLASMA

G. Firrao¹, M. Moretti¹, M. Ruiz Rosquete¹, E. Gobbi^{1,2} and R. Locci¹

¹ Dipartimento di Biologia Applicata alla Difesa delle Piante, Università di Udine, 33100 Udine, Italy

² Biodiversity s.r.l., Via Corfù 71, Brescia, Italy

SUMMARY

Nanobiotransducers are biomolecules linked to nanostructures with the ability to generate a physical signal upon the occurrence of a molecular event of biological interest. In this paper we report the use of a diagnostic probe made of a specific oligonucleotide bearing a fluorescein at its 5' end and a 2 nm gold particle at its 3' end, which acts as a quencher. The nanobiotransducer performs as a molecular beacon and emits a stronger fluorescence signal when hybridised to target DNA. The advantage over conventional molecular beacons is that this approach does not require any sequence constraint, because the proximity between the emitter and the quencher is due to the physical adsorption of the fluorophore on gold and not to the formation of DNA hairpins.

The probe was used to confirm the identity of PCR amplifications obtained from DNA extracted from grapevine plants affected by flavescence dorée. The nanobiotransducer detected all positive samples, although the quantitative results were not in strict agreement with the densitometry values obtained in parallel slot-blot membrane hybridisation experiments carried out with a biotin-labelled probe. This appears to be the first report of the use of a nanobiotransducer for the detection of an infectious agent in field samples.

Key words: self-assembled nanoparticles, fluorescence, diagnosis, DNA, probe.

INTRODUCTION

Most diagnostic assays for detecting the phytoplasmas are carried out using nucleic acids technology. PCR amplification of the 16S rDNA gene, introduced by Deng and Hiruki (1991), performed exceptionally well in this context and it is now regarded as the method of choice. Nevertheless, the DNA based assays are time

consuming and relatively expensive, and this hampers their wider use in the agro-industry. In terms of efficiency and cost, there are two major bottlenecks in the DNA based detection of phytoplasmas, and both have been the subjects of continuous study. First, the nucleic acids extraction is critical, since a relatively large amount of tissue needs to be processed and its DNA concentrated into a few microliters. Many DNA extraction procedures have been developed in the last 15 years, and are still being evaluated and improved (Kirkpatrick *et al.*, 1987; Ahrens and Seemüller, 1992; Lee and Davis, 1992; Firrao *et al.*, 1993a,b; Gibb and Padovan, 1994; Barba *et al.*, 1998; Zhang *et al.*, 1998; Palmano, 2001; Boudon-Padieu *et al.*, 2003). Second, characterization of the PCR product, strictly necessary in phytoplasma diagnostics where conserved genes are used as the target and single crops may be affected by several different but related pathogens, can be time consuming and laborious. At present, the post-PCR characterization is carried out by nested PCR with specific primers (Davis and Lee, 1993), endonuclease digestion followed by gel electrophoresis (Lee *et al.*, 1993), hybridisation to specific oligonucleotide probes (Firrao *et al.*, 1993a), PCR-ELISA (Poggi Pollini *et al.*, 1997), and real-time PCR (Baric and Dalla Via, 2004; Bianco *et al.*, 2004). Therefore the development of more efficient methods for the identification of PCR products would be of great value to encourage the use of molecular diagnostics in the agroindustry. In this paper we evaluate the potential of a modern hybridisation-based assay for the post-PCR processing of samples in the molecular diagnosis of phytoplasmas.

In a conventional DNA/DNA hybridisation assay, the nucleic acids to be identified are denatured and bound to solid support such as a nylon membrane, which is then dipped in a solution containing labelled probe molecules, which are complementary in sequence to the target. The labelled probe is allowed to interact with the nucleic acid to be identified and, if target sequences are present, to hybridise. The unreacted probe molecules are removed by washing and the label is visualized in order to reveal the presence of target sequences in the samples. As this is rather laborious, research has focused on developing of new hybridisation

formats, which could speed up the diagnostic assay, while maintaining its characteristics of specificity and sensitivity. A major breakthrough in the field was the development of probes, which change their detectable behaviour upon hybridisation to specific targets, named "molecular beacons". These single-stranded oligonucleotide probes form a stem-loop structure where one end of the probe is labelled with a fluorophore, and the other end is tagged with a quencher. In the presence of a complementary target, the probe hybridisation results in loss of the stem-loop structure, physical separation of fluorophore and quencher, and an increase in measurable fluorescence.

The advantages of molecular beacons are twofold. First, there is no need to wash out the excess unhybridised probe, as it does not contribute significantly to the signal strength. Secondly, and more important, there is no need to immobilize the nucleic acids on a solid support, as separation is not necessary. This eliminates the time consuming transfer and support-binding of the samples, and also positively affects the kinetics of the hybridisation, thus greatly improving the assay's speed and efficiency.

Dubertret *et al.* (2001) highlighted the ability of gold nanoparticles to act as fluorescence quenchers in molecular beacons. More recently, it was shown that gold nanoparticle probes are actually a further development of the art, as they solve a major drawback of the molecular beacons: Maxwell *et al.* (2002) synthesized a DNA oligonucleotide fluorescently labelled at its 5' end and conjugated it, at the 3' end, to a gold nanoparticle, thus obtaining a circular structure because the fluorescent reporter was physically adsorbed by the gold. In this condition, the probe did not fluoresce, being quenched by the gold nanoparticle. Upon hybridisation to a complementary oligonucleotide the probe fluorescence increased, conceivably due to the physical separation of the gold from the fluorescent reporter in the double stranded DNA hybrid. Even in the absence of nucleotide sequences determining the formation of hairpin structures, the gold-DNA-fluorophore structure acts as a molecular beacon, and can therefore be used in diagnostic procedures, particularly in cases where assays based on DNA analysis cannot be circumvented. The diagnosis of the phytoplasma associated with the *flavescence dorée* (FD) of grapevine is one of such cases.

Flavescence dorée is a widespread destructive phytoplasma disease of grapevine in Europe (Boudon-Padiou, 1999). At present its diagnosis is difficult because the symptoms are easily confused with those of other grapevine yellows, associated with phylogenetically distinct phytoplasmas, which are of less phytopathological concern. Moreover, phytoplasmas cannot be cultivated *in vitro*, and their concentration in infected tissues is usually low. The detection of the FD associated phytoplasma (FDp) is at present based on the amplification

via nested PCR of either a specific target DNA sequence (Angelini *et al.*, 2001; Clair *et al.*, 2003) or a fragment of the 16S rDNA gene (Bertaccini *et al.*, 1995). The success of the amplification reaction is then checked by digestion with restriction endonucleases and/or gel electrophoresis. Alternatively, the amplification product can be recognized by hybridisation to an oligonucleotide probe (Firrao *et al.*, 1999a, 1999b). The present work was carried out to evaluate whether this last step could be replaced by hybridisation to a gold-DNA-fluorophore structure according to Maxwell *et al.* (2002).

Those authors introduced the term nanobiotransducers to refer to biomolecules linked to nanostructures, which can generate a physical signal when a molecular event of biological interest occurs. Nanobiotransducers are a novel scientific curiosity, so far; to translate their potential into practical applications it is necessary to evaluate their performances with real samples.

MATERIALS AND METHODS

DNA extraction. Fresh grapevine leaves were processed with a modification of the procedure developed by Ahrens and Seemüller (1992), according to standard protocols (Pasquini *et al.*, 2001). The nucleic acid obtained from 2.5 g of veins was then resuspended in 100 µl of TE buffer.

PCR amplification. Total nucleic acids were amplified using a nested PCR protocol. A first pair of primers, P1 of Deng and Hiruki (1991) and P7 of Smart *et al.* (1996), was used to specifically amplify a large fragment including the 16S rDNA and 16S/23S spacer region of phytoplasmas. After 3 min at 94°C initial denaturation, 35 cycles of 1 min at 94°C, 90 sec at 55°C, 80 sec at 72°C were performed, followed by 5 min at 72°C final DNA extension. On a 1:100 dilution of the first amplification product, a second primer pair, R16(V)F1 / R16(V)R1 according to Lee *et al.* (1994), was then used to specifically amplify a 1100 bp fragment of the 16S rDNA from phytoplasmas belonging to the elm yellows group (which includes FDp).

Membrane hybridisation to biotin labelled probe. Standard hybridisation was performed as previously described by Malisano *et al.* (1996). Briefly, the products of nested PCR were loaded on a nylon membrane assembled in a slot-blot apparatus and were hybridised overnight with a FDp specific biotin labelled probe (FFlav: 5' - CTT GTT ATA GAA ACT GTC TTG AC - 3'; Firrao *et al.*, 1999a) corresponding to nucleotide positions 586-608 of the sequence accession X76560 (Seemüller *et al.*, 1994). After stringency washes, blocking, incubation with streptavidin-AP conjugate, washings and label

development, a colour precipitate formed in correspondence to positive PCR products. The intensity of signals was estimated by analysis with a QBASIC program (Firrao, unpublished) of a bitmap file obtained by scanning the wet membrane.

Nanobiotransducers preparation. Gold nanoparticles were prepared by chemical reduction of HAuCl_4 according to Maxwell *et al.* (2002), with modifications suggested by the authors. Briefly, 375 μl of a 4% (w/w) HAuCl_4 solution and 500 μl of a 0.2 M K_2CO_3 solution were added to 100 ml of distilled water in an ice bath with rapid stirring. With continued stirring, five 1 ml aliquots of a fresh 10 mM NaBH_4 solution were sequentially added. Reaction was completed stirring on ice for additional 15 min. Gold nanoparticles were quantified by measuring the absorbance spectrum of an aliquot. Typically, a 512 nm absorption peak, characteristic of gold nanoparticles about 2 nm in diameter and with a molar extinction coefficient (ϵ) of $6.5 \cdot 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ (Maxwell *et al.*, 2002), was detected.

A 5'-fluorescein labeled oligonucleotide with a 3'-SH group (named FflavFS) was synthesized (MWG Biotech, Germany) with the following sequence: 5'-Fluorescein - TTT TTT CTT GTT ATA GAA ACT GTC TTG AC TTT TTT - SH - 3'. The oligonucleotide probe was first reduced with dithiothreitol and purified through a Sephadex column (NAP5 column, Amersham Biosciences, Little Chalfont, England), then incubated overnight with gold nanoparticles at a molar ratio of 2:5:1. The oligo-particle solution was gradually aged in a PBS buffer, over a 16 h period (Demers *et al.*, 2000), then dialyzed against PBS (Slide-A-Lyzer, 3500MWCO, Pierce), aliquoted and stored at -20°C or kept at 4°C for up to a month to test their stability.

Nanobiotransduction assay. A commercial apparatus for real time PCR (Biorad iCycler; Hercules, CA, USA) was used for measuring fluorescence. Each DNA sample to be assayed was ethanol precipitated, washed, recovered by centrifugation and then resuspended in 10 μl of 10 mM Tris, pH 8.5. The nanobiotransducer was then added in amounts of 0.3, 1 or 3 pmol. Five μl of 20x SSC (1x SSC is 150 mM NaCl, 15 mM sodium citrate) were added and the volume was brought up to 50 μl with water. The samples were heated to 95°C to ensure complete denaturation and the fluorescence was measured (F_{denat}); then the samples were cooled slowly ($0.5^\circ\text{C}/\text{min}$) to room temperature (25°C) and the fluorescence was measured again (F_{hybr}). A value named F_{diff} was calculated as the ratio $F_{\text{hybr}}/F_{\text{denat}}$. Finally, F_{abs} was calculated as the difference between F_{diff} of each sample and F_{diff} of a negative control sample containing only the nanobiotransducer with no target DNA.

RESULTS

Preparation of nanobiotransducers. Gold nanoparticles were easily made and coupled to a fluorescein labeled oligonucleotide probe. The purification of the nanobiotransducers was however difficult to achieve. Standard ultracentrifugation (as suggested by Maxwell *et al.*, 2002) at various speeds, ultracentrifugation with a sucrose cushion, and ethanol precipitation produced a black pellet, which could not be resuspended. Therefore the DNA-gold constructs were purified by dialysis, although this method was poorly suitable for removing unreacted oligonucleotides. Further work is therefore needed to optimize this step. We believe that the gold particles should be stabilized in order to make them less prone to form aggregates.

Research carried out to date on nanobiotransducers is still restricted to hybridisation to synthetic DNA, comparing fluorescence emissions in the presence or absence of complementary oligonucleotides (Maxwell *et al.*, 2002). The analysis of 'real' DNA samples may be limited by the thermal instability of gold nanoparticles, given the high temperature required for the complete denaturation of double stranded DNA. Fig. 1 (solid line) shows a typical fluorescence response of nanobiotransducers heating to 95°C followed by cooling to 26°C . It can be seen that fluorescence increases when the temperature descends from 95°C to about $50\text{-}60^\circ\text{C}$ and then remains almost constant. We believe that the increase in fluorescence is due to the release of free fluorescein-labelled DNA following the destabilization of the gold nanoparticles at temperatures above $50\text{-}60^\circ\text{C}$. The thermal behaviour of nanobiotransducers is different in the presence of a complementary target oligonucleotide, which may hybridise. The broken line in Fig. 1 shows that in the presence of a complementary oligonucleotide the fluorescence continues to increase in the

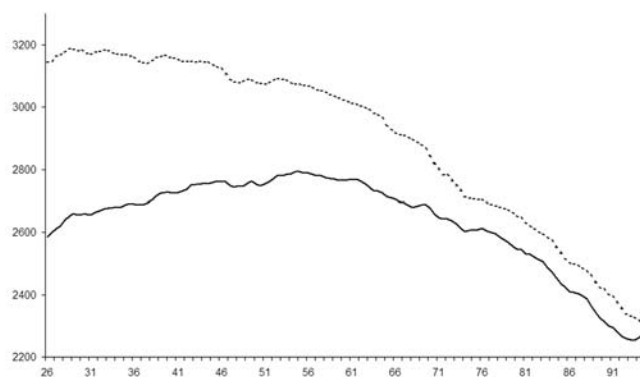


Fig. 1. Experimental thermal profile of the fluorescence emission intensity (arbitrary value scale) from solutions containing 10 pmol nanobiotransducer with (solid line) and without (broken line) the addition of 100 pmol of hybridising target oligonucleotide.

range from 50°C to 26°C. We believe that the fluorescence increase at temperatures below 50°C is due to the conformation change of the nanobiotransducer subsequent to the hybridisation. This differential behaviour may be used for detection purposes.

Specificity, sensitivity and stability of nanobiotransducers. The nanobiotransducer probes were assayed by DNA/DNA hybridisation with synthetic oligonucleotides. Fig. 2 shows the results of the hybridisation of different amounts of the nanobiotransducer with an excess of complementary and non-complementary oligonucleotides, carried out with freshly prepared, 30-day and 60-day-old nanobiotransducers. The figure shows that when the nanobiotransducers are used at concentrations of 0.3, 1 or 3 pmol, F_{abs} values from the samples containing target DNA complementary to the probe sequence are always higher than those from mismatching DNA. The discrimination power appears to be lower with nanobiotransducers kept at 4°C.

Diagnosis of field collected samples. Among 300 or so samples of grapevine with yellows symptoms processed from June to September 2003, 20 samples scored as flavescence dorée positive were chosen. When assayed by nested PCR, the nucleic acids extracted from infected plants produced a single amplicon of about 1,100 bp, although some samples gave relatively weak bands (result not shown).

Aliquots of the amplification products were loaded on a nylon membrane, and hybridised to a biotin-la-

belled oligonucleotide probe known to be specific for the FDp 16S rDNA (Firrao *et al.*, 1999a,b). Hybridisation results were quantified by densitometry, as shown in Fig. 3 (lower graph, arbitrary values). All samples produced a readily detectable hybridisation signal, in some cases stronger than others, but always at least 10 times higher than the non-target DNA sample.

The same amplification products were also individually hybridised to 1 pmol of nanobiotransducer probe. The resulting detected fluorescence values are shown in a graph in the upper panel of Fig. 3. All field samples produced a positive F_{abs} value, meaning that all had a fluorescence shift larger than the control. Although all F_{abs} values from field samples were at least twice those produced by the non-target DNA samples, some of the samples, which gave the strongest hybridisation signal in the slot blot (such as samples 3 and 7), performed relatively poorly in the nanobiotransducer assay. We do not have an interpretation for this result, although we suspect that the instability of the gold-DNA conjugate may have contributed to the generation of high noise values.

DISCUSSION

The results reported in this work show that nanobiotransducers, in conjunction with PCR, could be used to detect the flavescence dorée phytoplasma. This appears to be the first reported use of nanobiotransducers to detect an infectious agent in 'real' samples of either ani-

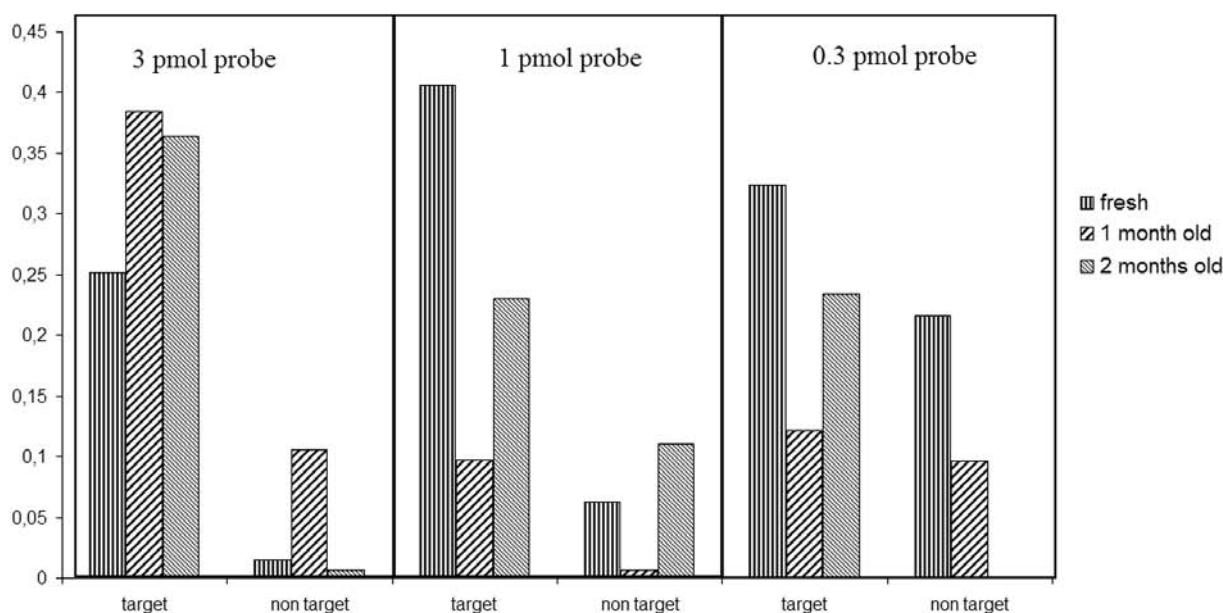


Fig. 2. Comparison of F_{abs} values obtained from 3 pmol, 1 pmol, and 0.3 pmol of nanobiotransducers freshly prepared or stored for 1 or 2 months at 4°C, in the presence of hybridising ("target") or not hybridising ("non target") oligonucleotides.

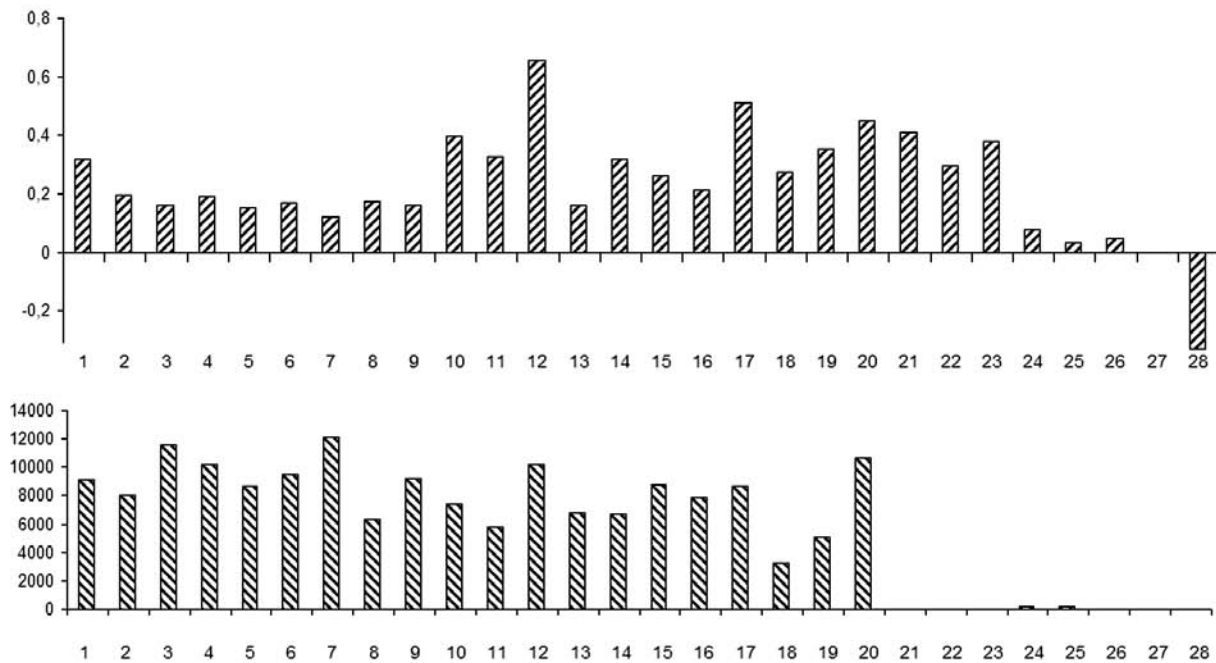


Fig. 3. Analysis of field collected samples: comparison of F_{abs} values from the nanobiotransducer assay (upper graph) vs. results of densitometric scanning of slot blot hybridisation (lower graph, arbitrary value scale). Bars 1-20: PCR products from field samples; bars 21-23: positive controls (hybridising oligonucleotides, used only in the nanobiotransducer assay); bars 24-26: negative controls (non-hybridising oligonucleotides in the nanobiotransducer assay, non-hybridising PCR products in the slot blot hybridisation assay); bar 27: no target control; 28: no probe control (only in the nanobiotransducer assay).

mals or plants. The potential of nanobiotransducers is great, since their use is extremely simple and rapid, compared to conventional techniques such as filter membrane hybridisation or gel electrophoresis. Although in this work the experiments were performed using a real time PCR machine, the actual hardware requirements are much simpler. A simple, blue-LED-diode-based reader is being developed in our laboratory, which costs much less than a real time PCR station. In addition, the nanobiotransducers can distinguish among similar amplicons, and might therefore be used to characterize samples, which reacted positively with phytoplasma-universal primers in direct PCR (M. Moretti, E. Gobbi and G. Firrao, unpublished results). Thus, a single amplicon could be assayed with several specific nanobiotransducers and the pathogen rapidly identified. This would be particularly valuable for grapevine yellows, which can be associated with at least five different phytoplasmas.

Nevertheless, there are still many aspects that limit the performance of the nanobiotransducers and prevent their introduction, in the present format, into routine diagnostics. First, it is difficult to purify the nanobiotransducers after conjugation of the gold nanoparticles with oligonucleotides. In our hands, only dialysis proved to be a workable method, but the resulting preparation was probably contaminated by unreacted oligonucleotides; the relatively high background of our

preparations may have been due to the unquenched fluorescence of unreacted fluorescein-labelled oligonucleotides. A possible way to limit the formation of aggregates of gold colloids, thus allowing ultracentrifugation, may be to stabilize the particles with citrate (Storhoff *et al.*, 1998) or polyamidoamines (Zheng and Dickson, 2002).

A second limitation is the relatively limited power of discrimination, compared with conventional techniques such as membrane hybridisation. In our assays, some of the positives gave a signal only twice that of the negatives. This limited power may be less relevant in the analysis of nested PCR products, but might be serious when sensitivity is required as for direct DNA analysis or poorly amplified PCR products. In addition, we did not find a direct relationship between the F_{abs} values and the signal intensities of the conventional slot-blot hybridisation. A likely reason is the limited stability (particularly at high temperatures) of the gold-DNA conjugate: when gold and DNA separate, fluorophore quenching is suppressed and this generates noise. It is therefore important to develop new chemical strategies to replace the gold-sulphide bond at present used as a link. Moreover, it has been reported that the quenching effect of gold nanoparticles is different with different fluorophores, fluorescein being one of the less effective (Dubertret *et al.*, 2001). Since a wide array of fluorescent functionalizations is available from commercial oligonucleotide

suppliers, a simple change of the label might give significant improvement. The recent report of Fan *et al.* (2003) that gold nanoparticles can very efficiently quench the fluorescence of light harvester polymers, such as polyfluorene, opened new perspectives in the development of the optical performances of nanobiotransducers.

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