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Colorimetric detection of eukaryotic gene expression with DNA-derivatized gold nanoparticles

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Abstract

Thiol-linked DNA-gold nanoparticles were used in a novel colorimetric method to detect the presence of specific mRNA from a total RNA extract of yeast cells. The method allowed detection of expression of the *FSY1* gene that encodes a specific fructose/H^+ symporter in *Saccharomyces bayanus* PYCC 4565. FSY1 is strongly expressed when the yeast is grown in fructose as the sole carbon source, while cells cultivated in glucose as the sole carbon source repress gene expression. The presence of FSY1 mRNA is detected based on color change of a sample containing total RNA extracted from the organism and gold nanoparticles derivatized with a 15-mer of complementary single stranded DNA upon addition of NaCl. If FSY1 mRNA is present, the solution remains pink, changing to blue-purple in the absence of FSY1 mRNA. Direct detection of specific expression was possible from only 0.3 μg of unamplified total RNA without any further enhancement. This novel method is inexpensive, very easy to perform as no amplification or signal enhancement steps are necessary and takes less than 15 min to develop after total RNA extraction. No temperature control is necessary and color change can be easily detected visually.

Keywords: Gene expression detection; RNA detection; Gold nanoparticles; Biosensors; Nanotechnology

1. Introduction

Utilization of thiol-functionalized-DNA attached to gold nanoparticles as nanoprobes for the detection of specific DNA sequences is a burgeoning technique (see, e.g. Storhoff et al., 2004a and references

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therein) as an inexpensive and easy to perform alternative to fluorescence- or radioactivity-based assays. Typically, a colloidal solution of gold nanoparticles with approximate diameters of 5–30 nm exhibits a red color due to the optical absorption peak around 520 nm caused by surface plasmonic resonance. Aggregation of the nanoparticles, induced by a cross-linking mechanism, shifts the absorption peak toward longer wavelength and the solution turns purple (Storhoff et al., 1998). Recently, a non-cross-linking DNA hybridization method was described, where aggregation of the oligonucleotide-functionalized gold nanoprobes is induced by an increasing salt concentration in the presence of complementary/mismatched oligonucleotides of the same size as the oligonucleotides of the probe (Sato et al., 2003). Both these principles have since been applied to DNA analysis, recurring to synthesized oligonucleotide sequences and/or PCR amplified products as targets.

Gene expression detection can provide powerful insights into the chemistry and physiology of biological systems. Better understanding of the molecular mechanisms underlying biological processes can be achieved by comparing gene expression between cells in different states or between cells from different tissues. Furthermore, an abnormally expressed gene can be used as a new drug target or as a genetic marker for diagnosis. Most mRNAs exist in very small quantities, needing accurate and extremely sensitive detection methods. Researchers often use model organisms where mRNA is easily available, providing better understanding of more complex organisms such as humans. Of all model systems, yeasts are particularly attractive because they include some of the simplest eukaryotic organisms, have easy growth conditions and quick reproduction times, thus providing large amounts of RNA (Botstein et al., 1997; Andrade et al., 1998; Stanchi et al., 2001; Oliver, 2002). We have chosen *Saccharomyces bayanus*, commonly used in the wine industry, as model organism and RNA source. *S. bayanus* strains harbor a gene, *FSY1*, encoding a specific fructose/H^+ symporter (Gonçalves et al., 2000). FSY1 expression is strongly regulated by the available carbon source in growth medium. FSY1 is abundantly expressed in cells grown up to 0.5% fructose but repressed at the same glucose concentration (Rodrigues de Sousa et al., 2004).

Reverse transcription-polymerase chain reaction (RT-PCR) has been considered the most sensitive technique for gene expression analysis. The technique consists of two steps: synthesis of cDNA from RNA by reverse transcription (RT) and amplification of the specific cDNA by polymerase chain reaction (Freeman et al., 1999; Bustin, 2000). The resulting amplified product can then be used in microarray analysis or hybridization assays. Even though these methods are used to analyze RNA expression, they are actually based on detection of amplified cDNAs or bound probes instead of the RNA itself. Direct detection of RNA, especially mRNA, has remained a challenge. Recently, an RNA detection method was described (Huber et al., 2004) that uses unamplified total human RNA allowing for differential gene expression detection from 0.5 μg of unamplified total RNA by means of gold nanoparticle probes after signal amplification by autometallography.

Here we report a new colorimetric method for the specific detection of gene expression through the use of Au nanoprobes, i.e. gold nanoparticles functionalized with thiol-DNA-oligonucleotides. The method uses non-cross-linking aggregation of Au nanoprobes to detect gene expression directly from total RNA extract after minimal make-up and without recurring to any detecting apparatus. The method enables specific mRNA detection in as little as 0.3 μg of unamplified total RNA and requires only minutes to be carried out. Furthermore, no amplifications or signal enhancement steps are necessary to allow detection, making it an extremely simple and fast method for direct detection of eukaryotic gene expression.

2. Materials and methods

2.1. Materials

All synthesized thiol modified oligonucleotides were purchased from MWG Biotech, Germany. All chemicals were from Sigma Aldrich and of the highest purity available.

2.2. UV–visible spectroscopy and photography

Absorption spectra were performed at 20 °C in a UNICAM, model UV2, UV–visible spectrophotometer equipped with a thermostated cell-holder. Quartz cells were from Hellma, Germany, Ultra-Micro with

10 mm path length and 100 μl volume. A 10 mM phosphate buffer with 0.1 M NaCl was used as reference. All photographs in this work were recorded with a Coolpix E950 Digital Camera (Nikon Corp., Japan).

2.3. Probe design and oligonucleotide purification

The probe sequence 5′-thiol-CCAAGCTACTATTTG-3′ was derived from the *FSY1* gene sequence (GenBank accession no. AJ250992). The thiolated oligonucleotide was resuspended in 1 ml of 0.1 M DTT and extracted three times with ethyl acetate. The oligonucleotide solution passed through a desalting NAP-5 column (Pharmacia Biotech, Sweden) for purification.

2.4. Preparation of the gold nanoparticle probes

Gold nanoparticles were prepared by the citrate reduction method described by Lee and Meisel (Lee and Meisel, 1982). Briefly, 250 ml of $HAuCl_4$ 1 mM were brought to a boil while stirring in a 500 ml round-bottom flask. Twenty-five millilitres of sodium citrate 38.8 mM were quickly added and the mixture was refluxed for 15 min with continuous stirring. The flask was let to cool to room temperature and stored in the dark until use. Gold nanoparticle concentration was determined by the Lambert–Beer law assuming a calculated molar absortivity for the plasmon resonance band maximum (526 nm) of 2.33×10^8 $M^{-1}\,cm^{-1}$. The synthesized colloidal gold nanoparticles presented an average size of 17 nm as determined from transmission electron microscopy (TEM) observations (data not shown).

The gold nanoprobes were synthesized by derivatizing 6 ml of an aqueous solution of gold nanoparticles (≈8.55 nM) with an appropriate volume of 10 μM thiolated oligonucleotide using a previously described protocol (Storhoff et al., 1998). Briefly, the thiol modified oligonucleotides were initially incubated with gold nanoparticles for at least 16 h. The solution was then brought to phosphate buffer (pH 7), 10 mM, 0.1 M NaCl using an adequately concentrated solution and allowed to stand for 40 h. After centrifugation, the precipitate was washed with 5 ml of phosphate buffer (pH 7), 10 mM, 0.1 M NaCl, recentrifuged and redispersed in 5 ml of the same buffer to a final concentration in gold particles of 9.7 nM. Gold nanoprobes were stored in the dark at 4 °C.

2.5. Yeast growth and total RNA isolation

A strain of *S. bayanus* PYCC 4565 was cultivated in Yeast Nitrogen Base (YNB) medium either with 0.5% (w/v) fructose or 0.5% (w/v) in glucose as the sole carbon source. Cells were grown at 30 °C, in an orbital shaker (Gallenkamp, UK) at 150 rpm and harvested in mid-exponential phase at $OD_{640nm} \approx 0.8$.

Isolation of total RNA was performed as previously described (Griffioen et al., 1996) with few modifications. Briefly, cells were harvested by centrifugation and then washed in 1 ml extraction buffer (0.1 M Tris–HCl; 0.1 M LiCl; 0.1 mM EDTA; pH 7.5), centrifuged and resuspended in 500 μl of lysis buffer (0.1 M LiCl; 0.5% Li-dodecyl sulfate (LiDS); 10 mM EDTA; pH 7.5). The cells were ruptured by vortexing following the addition of 0.5 g of glass beads and 400 μl phenol. Three phenol extractions were performed followed by two chloroform:isoamylalcohol (24:1) extractions. RNA was recovered by ethanol/sodium acetate precipitation and resuspended in RNase free double-distilled water. To ensure absence of DNA contamination, RNA was further precipitated by addition of an equal volume of 4 M LiCl. The total RNA was again ethanol precipitated, resuspended in RNase free double-distilled water and kept at −20 °C until use. RNA purity and concentration were determined by UV spectroscopy. The quality of the RNA extracts was suitable for Northern blot, as indicated below.

2.6. FSY1 expression in S. bayanus

In *S. bayanus* PYCC 4565, the presence of FSY1 mRNA can be detected when cells are cultivated in low fructose concentrations (0.5%). RNA purified from cells cultivated in 0.5% glucose, i.e. where FSY1 expression is repressed, was used as negative control. The presence of FSY1 mRNA as indicative of gene expression was confirmed by Northern blot (Rodrigues de Sousa et al., 2004). The obtained total RNA proved unequivocally the presence of FSY1 mRNA (data not shown) and represented a positive control for the method.

2.7. Hybridization and color detection

Assay solutions containing the Au nanoprobes and target RNA were prepared by mixing various concentrations of the appropriate total RNA (final concentration 50–200 $\mu g\,ml^{-1}$) of each extraction with the Au nanoprobe solution (final concentration 2.5 nM). After 5 min of denaturation at 95 °C, the mixtures were allowed to stand for 30 min and 5 M NaCl was added to achieve a final NaCl concentration of 2 M. Blank measurements were made in exactly the same conditions but replacing total RNA for an equivalent volume of 10 mM phosphate buffer. After 15 min at room temperature for color development, photographs were taken. Both mixtures and the blank were assayed by UV–visible spectroscopic measurements of the plasmon resonance.

Each assay was repeated at least five times for each RNA extract. Extracts were obtained from at least five different yeast cultivations grown on different days. The obtained results were consistently as presented below.

3. Results and discussion

3.1. Gold nanoprobe stability in solution

We investigated the aggregation behavior of non-functionalized gold nanoparticles and of single stranded DNA-functionalized gold particles (Au nanoprobes) with increasing NaCl concentration. A solution of non-functionalized gold nanoparticles aggregated instantaneously after NaCl addition at low salt concentrations (0.1 M NaCl), which is revealed by a color change of the solution from red to deep purple (Kreibig and Genzel, 1985). Within a couple of minutes of salt addition, purple particles adsorbed to the tube wall and precipitated. In contrast, Au nanoprobes retained their original pink color for NaCl concentrations up to 1.5 M. At 2.0 M NaCl, a clear pink to blue-purple color change was observed resulting from nanoparticle aggregation. This color change is visually clear and can be followed by UV–visible spectroscopy. In fact, Au nanoprobes present a plasmon resonance at 530 nm for the pink non-aggregated form that red shifts to 650 nm (blue-purple) upon nanoprobe aggregation in solution (Kreibig and Genzel, 1985). Precipitation of the aggregated Au nanoprobes was observed to be much slower than for the case of the non-functionalized gold nanoparticles and occurs within 1–2 h after NaCl addition. This increased stabilization in solution for the single stranded DNA-functionalized gold nanoprobes compared to the non-functionalized gold nanoparticles has been previously observed (Sato et al., 2003) and it has been proposed to be due to an increased repulsion between the particles derived from the electrostatic charges of the exposed bases of single stranded DNA (Storhoff et al., 2002).

3.2. FSY1 expression detection with gold nanoprobes

FSY1 expression detection was performed with an experimental setup consisting of several total RNA extracts from yeast grown in two different conditions (FSY1 expression or repression) and Au nanoparticles functionalized with a complementary sequence to the FSY1 mRNA. The method consists on visual and spectrophotometric comparison of the solutions before and after salt induced Au nanoprobe aggregation: (i) “Blank”, the Au nanoprobe alone; (ii) negative control, hereafter designated as “FSY1neg”, containing total RNA from cells grown in 0.5% glucose as sole carbon source, in which FSY1 expression is repressed; and (iii) positive result sample, hereafter designated as “FSY1pos”, containing total RNA extracted from cells grown in 0.5% fructose as sole carbon source, where FSY1 is abundantly expressed. Table 1 illustrates the characteristic results of the method as determined visually. After NaCl addition, both the Blank and FSY1neg turn from the initial pink to a blue-purple color, denot-

Table 1
Qualitative method results after NaCl addition ([NaCl] = 2 M), as estimated visually

Sample	Color change from pink/ time to develop	Precipitation
FSY1pos	Darker pink/15 min	None
FSY1neg	Blue-purple/15 min	Some
Blank	Blue-purple/instantaneous	Extensive

All three samples were pink before NaCl addition. “FSY1pos” refers to a sample containing total RNA from cells that express FSY1, “FSY1neg” refers to a sample containing total RNA from cells that repress FSY1 expression and “Blank” refers to a control without RNA.

ing Au nanoprobe aggregation. Differences between these samples are related to the color changing rate, with the RNA-containing samples taking 15 min to develop full color whereas the Blank sample changes instantaneously after addition of the salt. Also, the aggregated particles from the Blank start to slowly precipitate immediately, whereas FSY1neg presents a much slower Au nanoprobe precipitation process, taking several hours to precipitate completely. In the case of positive samples, FSY1pos, the solutions maintain their original pink color after NaCl addition. In these samples, no precipitation was observed even after standing overnight at room temperature.

The detection assay was repeated using a serial dilution of RNA ranging from 0.2 to 0.5 μg of total RNA. Even though most assays exhibited a clear color distinction between FSY1pos and FSY1neg, best reproducibility of the assay was achieved with an amount of total RNA in the range 0.3–0.5 μg. Below that range, the difference in color between FSY1pos and FSY1neg was not discriminating.

In order to obtain a more detailed picture of the method, UV–visible spectrophotometry was used. Fig. 1 shows the spectrophotometric change 15 min after increase of NaCl concentration to 2 M, in solutions containing Au nanoprobes alone (Blank) or in the presence (FSY1pos) or absence (FSY1neg) of target mRNA. Aggregation of Au nanoprobes (pink to blue-purple color) seen in the Blank and FSY1neg samples is corroborated by UV–visible spectra, where an intense plasmon resonance band appears at 650 nm, with a concomitant decrease of the intensity of the original plasmon resonance at 530 nm (see Fig. 1). Such effect is not observed in positive samples (FSY1pos), where only a slight increase of the plasmon resonance band at 650 nm is observed. The overall spectrum is practically identical to the one obtained before salt addition, which is in agreement with the observed persistence of the pink color (see Table 1 and Fig. 1). Thus, the intensity of the plasmon resonance at 650 nm, a band resulting from Au nanoprobe aggregation, seems to be a convenient way to measure nanoprobe aggregation. Fig. 2 depicts the variation of the intensity of the plasmon resonance at 650 nm for both samples containing RNA in relation to the Blank sample, 15 min after addition of NaCl to all three samples for a final concentration of 2 M. Data was collected from UV–visible spectra of samples 15 min after NaCl addition and corrected

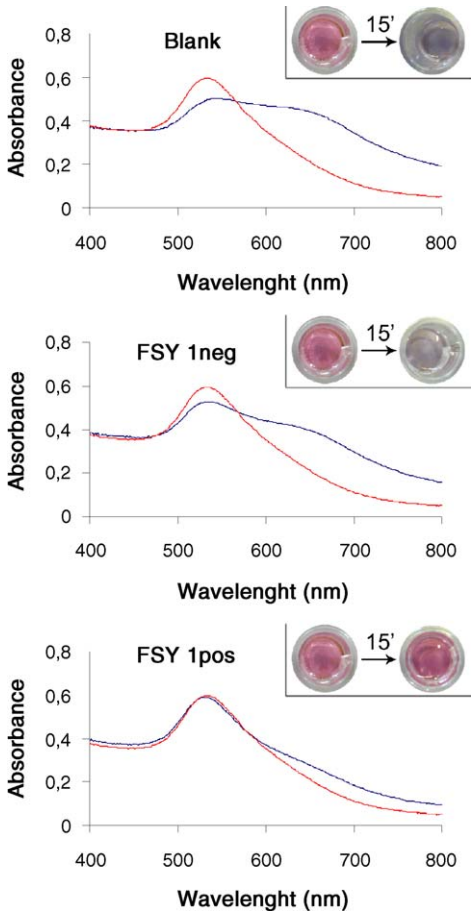

Fig. 1. FSY1 mRNA colorimetric detection method. The inset in each panel represents sample color change after 15 min of adding a concentrated NaCl solution for a final concentration of 2 M. UV–visible spectra were taken before (red trace) and 15 min after (blue trace) addition of the NaCl solution. "Blank" refers to nanoprobe alone; "FSY1pos" refers to sample containing total RNA from cells grown in 0.5% fructose, in which FSY1 mRNA expression occurs; "FSY1neg" is a negative control containing total RNA from cells grown in 0.5% glucose, in which FSY1 mRNA expression is repressed.

for Blank absorption. It can be observed that both the Blank and the FSY1neg show extensive aggregation and both present approximately 100% aggregation. The apparent excessive variation of FSY1neg (114%) can be explained by the extensive Au nanoprobe precipitation occurring in the Blank sample (see Table 1). Therefore, the measured absorbance after aggregation in the Blank is somehow smaller than that in FSY1neg.

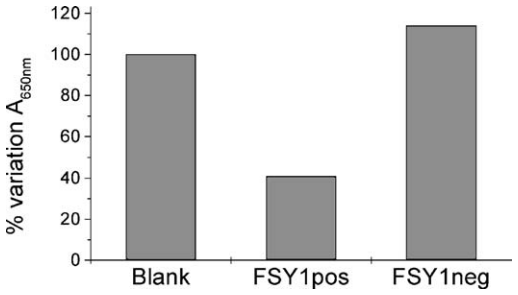

Fig. 2. Nanoprobe aggregation as measured by variation of the intensity of the 650 nm plasmon resonance for the three assay mixtures after 15 min incubation with [NaCl] = 2 M. Acronyms have the same meaning as in Fig. 1. The 650 nm peak corresponds to nanoprobe aggregation (see text for details).

Conversely, in the FSY1pos only 41% aggregation is observed. Nonetheless, this level of aggregation does not seem to influence greatly the initial pink color.

It is known that nucleic acid sequences protect gold nanoparticles against aggregation, possibly through electrostatic interactions between the negatively charged phosphate groups of the nucleic acid and the highly polarizable gold nanoparticles (Sandström et al., 2003). This premise can be used to interpret the smaller level of precipitation observed for FSY1neg (containing RNA sequences) when compared with the Au nanoprobes alone (Blank). The presence of nucleic acid in solution may act as a "buffer" for the increasing ionic strength, thus rendering the Au nanoprobes more stable. Following the same hypothesis, the increased stability observed for the complementary target (FSY1pos) might be explained by the added effect of this buffering plus the fact that all the Au nanoprobes hybridized with the complementary target mRNA. The resulting duplex should act like an "anchor" that keeps Au nanoprobes linked to the nucleic acid network, preventing their salt induced precipitation. In a recent report of specific DNA sequence detection using a non-cross-linking system, Sato et al. (Sato et al., 2003) describe aggregation of the nanoparticles upon hybridization with complementary targets in the presence of high salt concentration. It should be noted, however, that the targets are small complementary/mismatched oligonucleotides with the same length as the nanoparticle-linked oligonucleotides and therefore the characteristics of the system in terms of buffering and relative amounts of specific versus total nucleic acid are very different from the total RNA system presented here. In the system presented here, the specific target mRNA is only a minute fraction of the total nucleic acid in solution and the much longer sequence used as target probably improves the buffering effect.

Recently, another gold nanoparticle-based gene expression analysis system was presented (Huber et al., 2004) that also uses unamplified total RNA samples as the target nucleic acid. This method is substantially more complicated than the method described here as signal amplification by autometallography is used, leading to approximately 1000-fold increase of the detection signal (Storhoff et al., 2004b). Moreover, the detection of the scatter light is made with a specifically designed photosensor coupled with an imaging system, whereas in the method presented here, color change can be directly observed without any detection system or simply recurring to a common spectrophotometer.

In conclusion, a novel method for detection of eukaryotic gene expression is presented. The method is based on DNA-derivatized gold nanoparticles able to specifically detect FSY1 expression. Indication of FSY1 mRNA expression is obtained within 15 min by a color change of a solution containing total RNA extracted from yeast and a FSY1 specific Au nanoprobe. This method enabled specific mRNA detection from as little as 0.3 μg of unamplified total RNA and avoided the RNA to cDNA conversion step normally utilized by other methods. The inexpensive experimental setup, the short developing time without the need of signal amplification or temperature control and the fact that color change can be assessed visually are of great advantage and may prove to be valuable tools in real-time gene expression studies. Studies are underway in order to establish a quantitative relationship between signal intensity and gene expression level, as well as application of the method to other target genes in different organisms.

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