RNA quantification using gold nanoprobes -
application to cancer diagnostics

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Abstract

Molecular nanodiagnostics applied to cancer may provide rapid and sensitive detection of cancer related molecular alterations, which would enable early detection even when those alterations occur only in a small percentage of cells. The use of gold nanoparticles derivatized with thiol modified oligonucleotides (Au-nanoprobes) for the detection of specific nucleic acid targets has been gaining momentum as an alternative to more traditional methodologies. Here, we present an Au-nanoparticles based approach for the molecular recognition and quantification of the BCR-ABL fusion transcript (mRNA), which is responsible for chronic myeloid leukemia (CML), and to the best of our knowledge it is the first time quantification of a specific mRNA directly in cancer cells is reported. This inexpensive and very easy to perform Au-nanoprobe based method allows quantification of unamplified total human RNA and specific detection of the oncogene transcript. The sensitivity settled by the Au-nanoprobes allows differential gene expression from 10 ng/µl of total RNA and takes less than 30 min to complete after total RNA extraction, minimizing RNA degradation. Also, at later stages, accumulation of malignant mutations may lead to resistance to chemotherapy and consequently poor outcome. Such a method, allowing for fast and direct detection and quantification of the chimeric BCR-ABL mRNA, could speed up diagnostics and, if appropriate, revision of therapy. This assay may constitute a promising tool in early diagnosis of CML and could easily be extended to further target genes with proven involvement in cancer development.

Background

The National Cancer Institute envisions that over the
next years, nanotechnology will result in significant
advances in early detection, molecular imaging, targeted
and multifunctional therapeutics, prevention and control
of cancer [1]. Nanodiagnostics is a burgeoning field as
more and improved techniques are becoming available
for clinical diagnostics with increased sensitivity at lower
costs [2-10]. Due to their optical properties, gold nano-
particles (AuNPs) have been used for DNA/RNA
screening approaches, namely via functionalization with
thiolated oligonucleotides (Au-nanoprobes), capable of
specifically hybridizing with a complementary oligonu-
cleotide sequence [9].

The surface plasmon resonance (SPR) of AuNPs is
responsible for the intense colors - monodisperse Au-
nanoprobes (≈ 13 nm) appear red and exhibit a narrow
SPR band centered around 520 nm; a solution
containing aggregated Au-nanoprobes appears blue, due
to a red shift of the SPR. Our method relies on visual
and/or spectroscopy comparison of solutions before and
after salt induced Au-nanoprobe aggregation -presence
of complementary target prevents aggregation and the
solution remains red (SPR peak at ± 520 nm); non-com-
plementary targets do not prevent Au-nanoprobe aggre-
gation, resulting in a visible change of color from red to
blue (red-shift of the SPR peak to 600-650 nm) [5-7].
The principle of gold nanoparticles assay method detec-
tion of RNA hybridization is depicted in Figure 1. This
non-cross-linking method has already been successfully
applied for detection of eukaryotic gene expression with-
out reverse transcription or PCR amplification steps [6],
and for Mycobacterium tuberculosis detection [7,8].

Chronic myeloid leukemia (CML) is a clonal neoplastic
disease of the hematopoietic stem cell, whose hall-
mark molecular event is the genetic t(9;22)(q34;q11)
translocation known as the Philadelphia (Ph) chromo-
some [11,12]. This translocation - ABL gene (chromo-
some 9) and BCR gene (chromosome 22) - originates a
BCR-ABL fusion gene, leading to the expression of a

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chimeric BCR-ABL protein with tyrosine-kinase activity [13-15]. The most commonly used procedures for the initial diagnosis and management of CML patients are expensive and time consuming, e.g. karyotype analysis, reverse transcriptase polymerase chain reaction analyses (RT-PCR) and fluorescence in-situ hybridization (FISH) [16-18]. Therefore, there is a need for molecular methods able to detect and quantify the BCR-ABL fusion transcripts, which is of paramount relevance when monitoring minimal residual disease and genetic recurrence in patients known to harbor the translocation [19,20].

Here we present an Au-nanoprobe based approach for the molecular recognition and quantification of BCR-ABL b3a2 (e14a2) fusion for the early diagnosis of CML, which is inexpensive very easy to perform and uses total human RNA as target without reverse transcription and/or amplification.

Methods

Probe design and Au-nanoprobe synthesis

The probe sequence 5’-thiol-CGCTGAAGGGCTTTTGAACT-3’ and the complementary target derive from the BCR-ABL b3a2 (e14a2) chimeric protein mRNA (Gene-Bank accession no. AJ 131466.1: 5’-TGGATT-TAAGCAGAGTTCAAAAGCCCTTCAGCGGCCAGTA-3’), and the control oligonucleotide target sequences: BCR (Gene-Bank accession no. NM 005157.3: 5’-CTCCAGCTTTATCTGGAAAG AAGCCCTTCAGCGGCCAGTA-3’) and an unrelated target (5’-AGGAAAACGATTCCCTTCTAACAGAAATG TCCTGAGCAATC-3’). The way these sequences relate to each other is illustrated in Figure 2.

The 13 nm gold nanoparticles were prepared by the citrate reduction method described by Lee and Meisel [21]. The thiolated oligonucleotide was dissolved in 1 ml of 0.1 M DTT, extracted three times with ethyl acetate, and further purified through a desalting NAP-5 column (Pharmacia Biotech, Sweden) according to the manufacturer’s instructions. The Au-nanoprobe was prepared as described in Baptista et al [5]. Briefly, 500 μl of 10 μM thiol modified oligonucleotide was initially incubated with 6 ml of an aqueous solution of AuNPs (≈8.55 nM) for at least 16 h. After centrifugation (20 min at 14500 G), the oily precipitate was washed with 5 ml of 10 mM phosphate buffer (pH 8.0), 0.1 M NaCl, recentrifuged and redispersed in 5 ml of the same buffer to a final concentration in AuNPs of 8.5 nM. The resulting Au-nanoprobe was stored in the dark at 4°C.

Cell culture and total RNA isolation

K562 erythroleukemic cells (BCR-ABL positive cell line derived from CML patients in blast crisis) and HL-60 cell line, a human leukemic promyelocytic cell line (BCR-ABL negative) were cultured in 90% RPMI 1640 and 10% FBS at 37°C with 5% CO₂. Saccharomyces cerevisiae cells were grown in YPD medium at 30°C.
overnight. Human peripheral blood mononuclear cells (PBMC) from control individuals were separated from 3 ml of heparinized peripheral venous blood by Ficoll gradient (Histopaque®-1077, Sigma-Aldrich, St. Louis, USA) according to manufacturer’s specifications. Isolation of total RNA was performed using a High Pure RNA Isolation Kit (Roche Applied Science) according to the manufacturer’s protocol. RNA concentration was determined by UV photometry and the RNA was stored at -80°C until use. RNA integrity was evaluated on a 1% agarose gel stained by GelRed™.

Reverse transcription (RT) and PCR amplification
Total RNA extracted from K562 cells was subjected to RT with Revert-Aid™ M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania) according to the manufacturer’s specifications, using 20 μM of BCR-ABL reverse primer, annealing at 42°C for 1 h and 70°C for 10 min to inactivate the reverse transcriptase. The reverse transcription reaction product, a 273-bp fragment of the human BCR-ABL fusion gene (b3a2 junction), was PCR amplified using primers BCR-ABLforward (18 nt): 5'-AGTCTCCGGGGCTCTATG-3' and BCR-ABLreverse (20 nt): 5'-GATTATAGCCTAAGACCCGG-3'. PCR amplification of the b3a2 region was carried out in 25 μl using 0.25 μM of primers, 0.2 mM dNTPs with 1 U Taq DNA polymerase (Amersham Biosciences, GE Healthcare, Europe, GmbH). The PCR reactions were performed in duplicate on a MyCycler Thermocycler (Bio-rad). Thermal cycling conditions consisted of denaturation at 95°C for 5 min and 30 cycles of amplification, each cycle consisting of denaturation of 95°C for 30 s, annealing at 52°C for 30 s, elongation was at 72°C for 30 s and final elongation at 72°C for 5 min and cooling at 4°C. The sequence of the PCR products was confirmed by sequencing.

Real-Time RT-PCR assay
The Real-Time PCR amplification was performed in a Corbett Research Rotor-Gene RG3000 using SYBR GreenER Real-Time PCR Kit (Invitrogen, Karlsbad, CA, USA) according to manufacturer's specifications in 50 μl reactions containing cDNA from K562 and HL-60 cell lines, 1× SYBR Green SuperMix and 200 nM of BCR-ABLforward and BCR-ABLreverse. The amplification conditions consisted of 50°C for 2 min hold, 95°C during 10 min hold, followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. All the results were originated from three independent experiments.

Au-nanoprobe hybridization and color detection
The Au-nanoprobe assay was performed in a total volume of 30 μl containing the Au-nanoprobe at a final concentration of 2.5 nM, the appropriate targets
at a final concentration of 100 fmol/μl (100% complementary BCR-ABL target; 50% complementary BCR and ABL targets, and 100% non-complementary target) in 10 mM phosphate buffer (pH 8.0). Total RNA was used at a final concentration 10-60 ng/μl [100% complementary K562 cells RNA (BCR-ABL Positive); non-complementary HL-60 cells RNA (BCR-ABL Negative)]. Blank measurements were made in exactly the same conditions but replacing target or total RNA for an equivalent volume of 10 mM phosphate buffer (pH 8.0).

Following 5 min of denaturation at 95°C, the mixtures were allowed to stand for 30 min at 25°C and 0.3 M MgCl2 was added at a final concentration of 0.16 M. The ratio between the free and aggregated fractions after 15 min incubation with [MgCl2] = 0.16 M. The ratio between the areas under the curve of the SPR was calculated using the trapezoidal rule - $\text{AUC}_{500 \text{ nm}-560 \text{ nm}} / \text{AUC}_{570 \text{ nm}-630 \text{ nm}}$. A ratio of 1 may be considered as the point of equilibrium between non-aggregated and aggregated nanoprobe, hence the threshold to respectively consider the positive and negative discrimination of sequences (positive identification of complementary target ratio >1). Commonly, for discriminating between two significantly different aggregation levels, as for example in a YES/NO for identification of a given target, the ratio between the peaks at 520 nm and 600 nm is usually used. However, for identifying small differences in aggregation levels between two quantities for the same target, there is a need to decrease the noise level in the spectra. When establishing a ratio between two absorbance values, the error increases mainly due the noise in the spectra, which can be overcome (i.e. strongly reduced) by using an integral of the signal, i.e. the area under the curve.

The Au-nanoprobes were then used for the detection of the BCR-ABL b3a2 fusion mRNA in total RNA extracted from K562 cells (BCR-ABL positive cell line), HL-60 cells (BCR-ABL negative cell line), human peripheral blood mononuclear cells (PBMC) and S. cerevisiae cells - Figure 3B. Total RNA from HL-60 cell line and PBMC only express the normal BCR and ABL transcripts, which are 50% complementary to the probe sequence. Total RNA from an unrelated organism (S. cerevisiae) was used to confirm specificity of the detection method. The results originate from a minimum of three individual parallel hybridization experiments. BCR-ABL fusion discrimination was observed only for samples containing the complementary RNA target (K562 cells). Samples containing the normal BCR and ABL genes showed a minor stabilization of the Au-nanoprobe, yet below the threshold for positive identification of the target (ratio <1).

### Gold nanoprobe assay for RNA quantification

Once the specific identification of the target sequence was achieved, the Au-nanoprobes were used to evaluate both the limit of detection and quantification potential. For this purpose, different concentrations of the specific synthetic oligonucleotide target were used to spike in 20 ng/μl of total RNA extracted from the BCR-ABL negative cell line HL-60. Our data indicate a linear correlation ($R^2 = 0.9966$) between the $\text{AUC}_{500 \text{ nm}-560 \text{ nm}} / \text{AUC}_{570 \text{ nm}-630 \text{ nm}}$ for target concentration range between 33 and 133 fmol/μl (Figure 5). A non-complementary target was used in a parallel spike in
Figure 3 Au-nanoprobe detection of the BCR-ABL fusion gene sequence. (A) Colorimetric assay (above) and respective spectrophotometry (below) relative to the detection of synthetic BCR-ABL oligonucleotide target. Oligonucleotides with BCR or ABL sequence only (showing 50% complementarity) were used as controls and an unrelated target (showing 100% non-complementarity to the Au-nanoprobe) as negative control. (B) Detection of BCR-ABL in total RNA from K562 cell line, HL-60 cell line and human PBMC (harboring 50% complementary targets to the nanoprobe) and S. cerevisiae cells (100% non-complementary). Nanoprobe aggregation as measured by ratio of AUC500 nm-560 nm/AUC570 nm-630 nm. The dashed line represents the threshold of 1 considered for discrimination between Positive and Negative. The error bars represent the standard deviation from three independent assays.
experiment, where extensive aggregation of the Au-nanoprobe was observed for all tested concentrations.

In order to validate the detection and quantification potential of the Au-nanoprobes in the positive cell line (K562), Real-time RT-PCR was used. Our method showed a linear correlation for \( \text{BCR-ABL} \) detection within the range of 10-60 ng/\( \mu \)l of total RNA (see Figure 6). A linear association (\( R^2 = 0.9171 \)) was found between the two methods, Real-Time RT-PCR and Au-nanoprobe, for \( \text{BCR-ABL} \) detection (inset in Figure 6). Real-Time RT-PCR is a more robust and sensitive technique but time consuming, more expensive and requiring expensive equipment and highly trained personnel.

**Conclusions**

In conclusion, we demonstrated the potential of an Au-nanoprobe based assay for the specific identification and quantification of aberrant expression of genes involved in malignant diseases.
**Figure 5** Quantification of **BCR-ABL** by Au-nanoprobe. Ratio $\text{AUC}_{500\text{ nm}-560\text{ nm}}/\text{AUC}_{570\text{ nm}-630\text{ nm}}$ as function of specific target concentration in mixtures of 20 ng/µl total RNA from **BCR-ABL** negative cell line HL-60 spiked in with increasing concentrations of the synthetic oligonucleotide (black diamond’s - complementary target; blank squares - non-complementary target). The error bars represent the standard deviation from three independent assays.

**Figure 6** Au-nanoprobe based quantification of **BCR-ABL** fusion mRNA directly in total RNA extracted from K562 cell line. Nanoprobe aggregation as measured by ratio of $\text{AUC}_{500\text{ nm}-560\text{ nm}}/\text{AUC}_{570\text{ nm}-630\text{ nm}}$ for increasing concentrations of total RNA from a **BCR-ABL** positive cell line (K562) - 10 to 60 ng/µl. **Inset**: Real-Time RT-PCR vs. Au-Nanoprobe Assays. A linear association ($R^2 = 0.9171$) was found between the two methods. The error bars represent the standard deviation from three independent assays.
in cancer development. This Au-nanoprobe strategy allowed for detection of less than 100 fmol/μl of a specific RNA target, with the possibility of discriminating between a positive and negative from as little as 10 ng/μl of total RNA. As proof-of-concept we used the BCR-ABL fusion product that is of paramount importance in chronic myeloid leukemia, showing the application potential in cancer diagnosis. To our knowledge, this is the first report on quantification of human mRNA directly from total RNA without reverse transcription or amplification. The assay has a total work-up time of less than 45 minutes with comparable sensitivity to those demonstrated by traditional molecular biology methodologies.

List of Abbreviations

(CML): Chronic myeloid leukemia; (AuNPs): Gold nanoparticles; (Au-nanoprobes): Gold nanoparticles; (SPR): Surface plasmon resonance; (Ph) chromosome: Philadelphia; (PBMC): Peripheral blood mononuclear cells; (AUC): Area under the curve.

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Authors’ contributions
JC participated in the sequence alignment and design of the nanoprobe, carried out the nanoprobe synthesis, and performed the detection assays. JF participated in the design of the study. PB conceived the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References
1. National Cancer Institute: [http://nano.cancer.gov/].
2. Mirkin CA, Letsinger RL, Mueller RC, Storhoff JJ: A DNA-based method for rationally assembling nanoparomic into macroscopic materials. Nature 1996, 382(6592):607-609.
3. Storhoff JJ, Lucas AD, Garimella V, Bao YP, Muller UR: Homogeneous detection of unamplified genomic DNA sequences based on colorimetric scatter of gold nanoparticle probes. Nat Biotechnol 2004, 22(7):883-887.
4. Thaxton CS, Georganopoulos DG, Mirkin CA: Gold nanoparticle probes for the detection of nucleic acid targets. Clin Chim Acta 2006, 363(1-2):120-126.
5. Baptista P, Pereira E, Eaton P, Doria G, Miranda A, Gomes I, Quaresma P, Franco R: Gold nanoparticles for the development of clinical diagnosis methods. Anal Bioanal Chem 2008, 391(3):943-950.
6. Baptista P, Doria G, Henrique D, Pereira E, Franco R: Colorimetric detection of eukaryotic gene expression with DNA-derivatized gold nanoparticles. J Biotechnol 2005, 119(2):111-117.

7. Baptista PV, Kozioł-Montewka M, Paluch-Oles J, Doria G, Franco R: Gold-nanoparticle-probe-based assay for rapid and direct detection of Mycobacterium tuberculosis DNA in clinical samples. Clin Chem 2006, 52(7):1433-1434.
8. Costa P, Amaro A, Botelho A, Irácio J, Baptista PV: Gold nanoparticles assay for identification of mycobacteria from the Mycobacterium tuberculosis complex. Clin Microbiol Infect 2009.
9. Doria G, Franco R, Baptista P: Nanodiagnóstico: fast colorimetric method for single nucleotide polymorphism/mutation detection. IET Nanobiotechnol 2007, 1(4):53-57.
10. Griffin J, Singh AK, Senapati E, Lee E, Gayler K, Jones-Boone J, Rey PC: Sequence-specific HCV RNA quantification using the size-dependent nonlinear optical properties of gold nanoparticles. Small 2009, 5(7):839-845.
11. Hehlmann R, Hochhaus A, Baccarani M: Chronic myeloid leukemia. Lancet 2007, 370(9584):342-350.
12. Shet AS, Jahagirdar BN, Verfaille CM: Chronic myelogenous leukemia: mechanisms underlying disease progression. Leukemia 2002, 16(8):1402-1411.
13. Ren R: Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukemia. Nat Rev Cancer 2005, 5(3):172-183.
14. Wong S, Witte ON: The BCR-ABL story: bench to bedside and back. Annu Rev Immunol 2004, 22:247-306.
15. Mello J: Inviting leukemic cells to waltz with the devil. Nat Med 2001, 7(2):156-157.
16. Ou J, Vergilio JA, Bagg A: Molecular diagnosis and monitoring in the clinical management of patients with chronic myelogenous leukemia treated with tyrosine kinase inhibitors. Am J Hematol 2008, 83(4):296-302.
17. Apperley JF: Part I: mechanisms of resistance to imatinib in chronic myeloid leukemia. Lancet Oncol 2007, 8(11):1018-1029.
18. Burmeister T, Maurer J, Avadov M, Elmaagachi AH, Grunebauch F, Held KR, Hess G, Hochhaus A, Hoppner W, Lentes KU, Lubbert M, Schaffler K, Schlaflhausen P, Schmidt CA, Schuler F, Seege K, Seelig R, Thiede C, Vehrmann S, Weber C, Wilhelm S, Christmann A, Clement JH, Eberer U, Enzmann J, Leo R, Schleuning M, Schoch R, Theil E: Quality assurance in RT-PCR-based BCR/ABL diagnostics—results of an interlaboratory test and a standardization approach. Leukemia 2000, 14(10):1850-1856.
19. Beillard E, Pallisgaard N, van dV, W, Dee R, van der DE, Delabesse E, Macintyre E, Gottardi E, Saglioli G, Watzinger F, Lion T, van Dongen JI, Holland P, Gabert J: Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using ‘real-time’ quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) - a Europe against cancer program. Leukemia 2003, 17(12):2474-2486.
20. Gabert J, Beillard E, van dV, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cueva JM, Cave H, Pane F, Aerts JL, De MD, Thiron X, Pradel V, Gonzalez M, Vehrmann S, Malec M, Saglioli G, van Dongen JI: Standardization and quality control studies of ‘real-time’ quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 2003, 17(12):2318-2357.
21. Lee PI, Meisel D: Adsorption and surface-enhanced Raman of dyes on silver and gold rods. J Phys Chem 1982, 86(17):3391-3395.

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