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mRNA genotyping by gold nanoprobes

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RESUMO

A bionanotecnologia tem surgido como uma área com grande potencial para o diagnóstico molecular. Nomeadamente, a utilização de nanopartículas de ouro tem permitido o desenvolvimento de métodos de diagnóstico molecular mais sensíveis e com maior especificidade, a uma fracção do custo inerente aos testes convencionais.

No presente trabalho investigou-se a capacidade das nanossondas de ouro detectarem alvos com diferenças de uma só base em moléculas de RNA recorrendo ao método colorimétrico de *non-cross-linking*. Como *proof-of-concept* foram desenhadas, sintetizadas e caracterizadas nanossondas de ouro para a detecção de três SNPs (c.2732C>T, c.3232A>G e o c.3238G>A) presentes no gene *BRCA1,* um gene associado ao cancro da mama hereditário. Com o intuito de obter materiais de referência susceptíveis de utilização para calibração do método, fragmentos genómicos amplificados a partir de amostras biológicas contendo as sequências de interesse foram clonados em vector apropriado para posterior transcrição *in vitro/in vivo.*

Inicialmente, averiguou-se a capacidade das nanossondas detectarem oligonucletídeos sintéticos complementares, seguindo-se condições de maior complexidade através de *spiking* em RNA total de *Saccharomyces cerevisiae*. Por último, foram utilizados produtos de transcrição *in vitro/in vivo*. Foi possível detectar especificamente o alvo complementar em 0.12pmol/μL numa proporção de 0.25% de alvo complementar/RNA total. Foi também possível discriminar ambos os alelos do SNP c.3232A>G directamente em RNA utilizando apenas 0.08pmol/μL de transcrito sintetizado *in vitro*. No caso das amostras de transcritos *in vivo*, os resultados foram inconclusivos.

Palavras chave: Bionanotecnologia; ensaio *non-cross-linking*; nanossondas de ouro; genotipagem em RNA; *BRCA1*.

ABSTRACT

Bionanotechnology has emerged as a field with great potential for molecular diagnose. Namely, the use of gold nanoparticles has allowed the development of molecular diagnostic methods with greater sensitivity and specificity at a fraction of the cost inherent to conventional techniques.

The present work assessed the ability of gold nanoprobes to detect targets with single base differences in RNA molecules following the colorimetric non-cross-linking method. As proof-of-concept, gold nanoprobes were designed, synthesized and characterized to detect three different SNPs (c.2731C>T, c.3232A>G and c.3238G>A) in the *BRCA1* gene, a gene associated with inherited breast cancer.
Reference materials, susceptible to be used for the calibration of the method, were created by cloning genomic fragments amplified from biological samples containing the sequences of interest in an appropriate vector for subsequent in *vitro/in vivo* transcription.

Initially, the ability of target recognition by the gold nanoprobes was assessed using synthetic oligonucleotides targets alone and spiked-in total RNA of *Saccharomyces cerevisiae*, and later using transcripts synthetized *in vitro/in vivo.* This study revealed the capacity of target detection up to 0.25% of complementary target/total RNA, for a final concentration of complementary target of 0.12pmol/µL. It was also possible to detect and discriminate both c.3232A>G SNP alleles using only 0.08pmol/µL of *in vitro* transcript. For the in vivo transcript samples the results were inconclusive.

Keywords: Bionanotechnology; non-cross-linking assay; gold nanoprobes; RNA genotyping; *BRCA1*.

INDEX

FIGURE INDEX

TABLE INDEX

LIST OF ABBREVIATIONS

Abs - Absorbance

ARMS - Amplification Refractory Mutation System

AuNPs - Gold nanoparticles

Au-nanoprobes - Thiol-ssDNA modified gold nanoparticles

AuAg alloy nanoprobes - Thiol-ssDNA modified gold-silver nanoparticles

b - bases

bp - base pairs

CAGE - Cap Analysis of Gene expression

CCC - Critical Coagulation Concentration

DEPC - Diethyl pyrocarbonate

DMSO - Methyl Sulphoxide

DNA - Deoxyribonucleic acid

cDNA - Complementary Deoxyribonucleic acid

ssDNA - Single stranded Deoxyribonucleic acid

DTT - Dithiothreitol

ELOSA - Enzyme Linked Oligo Sorbant Assay

FISH - Fluorescent in situ Hybridization

GWAS - Genome wide association studies

IPO-FG - Instituto Português de Oncologia-Francisco Gentil

IPTG - isopropyl-beta-D-thiogalactopyranoside

LAMP - Loop Mediated Amplification

LB medium - Luria-Bertani medium

NPs - Nanoparticles

NTPs - Nucleosides triphosphate

dNTPs - deoxynucleotides triphosphate

OD - Optical Density

o/n - Over night

PCR - Polymerase Chain Reaction

PMAGE - Poly Multiplex Analysis of Gene Expression

eQTL - Expression Quantitative Trait Loci

RCA - Rolling Circle Amplification

RFLP - Restriction Fragment Length Polymorphism

RNA - Ribonucleic acid

mRNA - Messenger Ribonucleic acid

miRNA- Micro Ribonucleic acid

rRNA - Ribosomal Ribonucleic acid

tRNA- Transfer Ribonucleic acid

rpm - revolutions per minute

RT-PCR - Reverse Transcription Polymerase Chain Reaction

qRT-PCR - Real time Reverse Transcription Polymerase Chain Reaction

SAGE - Serial Analysis of Gene Expression

SDS - Sodium Dodecyl Sulphate

SMART - Signal Mediated Amplification of Ribonucleic acid Technology

SNP - Single nucleotide polymorphism

SOB medium - Super Optimal Broth medium

SOC medium - Super Optimal broth with Catabolite repression medium

SPR - Surface plasmon resonance

TEM - Transmission Electron Microscopy

TAE - Tris-acetate-EDTA

TBE - Tris-borate-EDTA

VNTR - Variable number of tandem repeats

YPD medium - Yeast Extract Peptone Dextrose medium

1. INTRODUCTION

1.1 From genome to transcriptome

The revealing of the human genome sequence in 2001 has given rise to a new post-genomic era with unprecedented benefits for the future of medicine and biotechnology[1]. Biomedical research has since focused on understanding the relationship between genetic factors, the environment and human traits. The big goal is to achieve a more efficient prediction, prevention, diagnosis and treatment of a disease, i.e. to reach a personalized medicine[2,3,4,5].

Genetic variability can be presented in many forms, including variable number of tandem repeats (VNTR), presence and absence of transposable elements, deletions, duplications, inversions and single nucleotide polymorphisms (SNPs)[6]. The latter is the most common one, being the cause of 90% of all variations in the human genome[7]. SNPs are single nucleotide variations within the genome that are present at least in 1% of a certain population[8], and provide a powerful resource as disease biomarkers in genome wide association studies (GWAS)[9].

Genetic variation can influence gene expression, both qualitative and quantitative, thus making it essential to characterize the transcriptome towards understanding the development of diseases. The transcriptome is the complete set of transcripts (e.g. mRNA, rRNA, tRNA, miRNA and many others) and their expression level within a cell, in specific physiological conditions [10]. SNP correlation in the transcriptome can be considered a quantitative trait. These correlation studies allow identification of genomic *loci* that regulate expression levels of mRNAs or proteins, known as expression Quantitative Trait Loci (eQTL)[11].

Few technologies have been developed for gene expression genotyping, such as enzymatic amplification and/or cleavage, hybridization and sequencing (Table 1.1). Most of these technologies need a first step of target DNA amplification provided by Polymerase Chain Reaction (PCR)[12,13]. In the case of RNA, it is required a prior conversion of RNA into its complementary DNA (cDNA) via a reverse transcriptase which is then amplified by common PCR, i.e. RT-PCR[14,15]. For mRNA quantification, a real time PCR can be performed via the generation of a fluorescent signal that can be correlated to the initial mRNA concentration in the sample, i.e. qRT-PCR[15,16]. Both PCR and real-time PCR techniques are usually expensive and time consuming, and are also error prone as most polymerases lack proof-reading activity. Nonetheless, PCR has been used to characterize single nucleotide variations, through allele specific PCR or amplification refractory mutation system (ARMS) approaches[17].

Target amplification is also possible through isothermal amplification methods[21], which enables DNA/RNA amplification without the thermocycling apparatus needed for PCR. Examples of these techniques are rolling circle amplification (RCA)[22], loop mediated amplification (LAMP)[23] and signal mediated amplification of RNA technology (SMART)[24]. These methods achieve amplification at a constant temperature using DNA polymerases with strong displacement activity such as *Pfu*, *Bst* and *Aac* polymerases. RCA involves only one primer, which can be also the template. The hybridization of the primer to a circularized DNA (probe) enables numerous rounds of isothermal enzymatic synthesis by continuously progressing around the circular DNA[22]. The LAMP technique in contrast to RCA, requires a set of four (to six) specific designed primers. The amplification is also assured by a polymerase with displacement activity only in the presence of the complementary target. This method has been applied to the characterization of RNA molecules by adding a first step of reverse transcription. Through this approach, Curtis *et al*.[26] managed to detect specific RNA sequences from HIV-1-infected individuals using blood directly from patients. More recently, using the same method, Li *et al*.[27] achieved real time detection and quantification of microRNAs in total RNA. Another isothermal amplification method is the SMART technique, which also enables detection of DNA or RNA targets by amplification of the signal of detection. It depends on two probes (extension and template), each having a complementary region to the target at adjacent positions to one another. When the probes encounter complementary target hybridization occurs and the DNA polymerase extends the extension probe by copying the template probe. This template probe possesses a T7 RNA polymerase promoter sequence. Only the double stranded sequence enables the activity of the T7 RNA polymerase, thus generating RNA copies. This technique has been used to detected gene expression by Wharam and co-workers[24], where they accomplished the detection of the SMART amplified signal through an Enzyme Linked Oligosorbent Assay (ELOSA).

Enzymatic cleavage is another genotyping approach based on the ability of certain enzymes, i.e. restriction enzymes, to cleave DNA by recognition of specific sequences. Restriction fragments length polymorphism (RFLP), can be used to characterize DNA/RNA once different sequences will have different patterns of cleavage by the specific restriction enzymes. The products can then be analyzed by gel electrophoresis[48]. This technique has the disadvantage of being dependent on the restriction enzymes available and thus to the restriction sequences. Another drawback is the impossibility to use RNA directly in RFLP analysis, since there are no known restriction enzymes for RNA cleavage[49]. Thus, genotyping RNA molecules by RFLP require a first step of RT-PCR to provide cDNA for enzyme recognition[28].

Another enzymatic method that allows the detection and quantification of mRNA, map mRNA termini and to determine the position of introns within the corresponding gene is the

Ribonuclease Protection assay. In this assay a high specific radiolabeled single stranded cRNA is synthesized and hybridized in excess to the target mRNA. Then a combination of RNaseA and RNaseT1 is used to digest the free mRNA and the digestion product is analyzed by polyacrylamide gel/microchip electrophoresis and autoradiography/phosphorImaging to reveal which mRNA was effectively protected from RNase digestion due to the formation of a duplex between the target mRNA and the cRNA probe[31, 32, 33].

Hybridization approaches are based on the capacity of hybridization between two complementary single stranded sequences and in the differences in stability of the formed double-strand. Examples of hybridization techniques for RNA analysis are Northern blot[34,35], fluorescent in situ hybridization (FISH) assays[36, 37,38] and Microarrays[39, 40]. Northern blot and FISH technologies are the only ones to directly detect RNA, by hybridization with labeled probes. FISH enables also the detection and localization of specific mRNA sequences *in vivo*. Microarray analysis is also based on hybridization specifically between the microarray probes and target sequences which are typically fluorescently labeled. The use of these platforms to quantify gene expression is achieved indirectly through hybridization between fluorescent labeled cDNA synthesized by RT-PCR and the microarray probes. However, the use of retro transcription for quantification of the RNA molecule does not allow the detection of subtle changes in gene expression. This is a big weakness once the levels of gene expression are very important to understand the biological process[45]. These techniques along with other hybridization techniques have some drawbacks typical of an hybridization method, such as the need for previous knowledge about the target DNA/RNA; high background noise due to cross hybridization; saturation of the signal; and, above all, limitations upon the hybridization efficiency that depends on the sequence[40].

Despite all the genotyping technologies available, sequencing is still the gold standard for genotyping, offering the highest degree of specificity and selectivity. During the last ten years a lot of improvements have been made in sequencing technologies. The main sequencing method is the Sanger method, based on electrophoresis of fluorescently labeled sequencing reaction products[50]. In the past 5 years plenty of new sequencing platforms have been developed, the so called “massively parallel” sequencing or high-throughput second generation sequencing increasing the data output, e.g. pyrosequencing[12].

The Sanger method firstly designed for DNA sequencing has been adapted to analyze gene expression giving rise to many gene expression sequencing techniques. These include Serial Analysis of Gene Expression (SAGE)[41], Cap Analysis of Gene Expression (CAGE)[42] and Poly Multiplex Analysis of Gene Expression (PMAGE)[43]. These analyses of RNA consist in synthesizing cDNA from the purified transcripts, which is then cutted by an anchoring enzyme,

usually *Nla*III. The linkers containing a restriction site sequence of a specific restriction enzyme that can cleave the cDNA in short tags are then joined to the cDNA. These tags are joined to form a chain, and undergo amplification by PCR, prior to sequencing. In the end it is possible to identity and characterize the absolute abundance of the analyzed transcripts. Moreover, the tags can be cloned, providing a library of that specific set of transcripts[44].

The most modern RNA sequencing technique is the so called RNA-seq, based on the new generation high-throughput sequencing platforms that were initially developed for the analysis of DNA[46]. Briefly, a population of RNA is converted to cDNA fragments with adapters attached to both ends. Each molecule with or without amplification is then submitted to high-throughput sequencing. This sequencing technique provides very important information about the transcriptome once it enables not only the detection but also the measurement of expression, by means of the absolute abundance of coding and non-coding transcripts. Other advantage of this high-throughput sequence method is the reduction of the required amount of RNA sample[45]. In fact, sequencing platforms are very important so as to identify previously uncharacterized genes and genes expression profiles of normal and pathological disease tissues[44,46].

Genotyping technologies are providing important information for the achievement of better diagnosis systems but current molecular diagnostic techniques are still out of reach for point-of-care diagnostic due to mainly 4 reasons: they are time consuming, require expensive instruments and technical expertise, and also require big amounts of biological samples[51,52].

Table 1.1 - Technologies for gene expression genotyping. The principle and their limit of detection are underlined.

Principle	Technology	Limit of detection**	Refs
Enzymatic Amplification	RT-PCR	1 pg - 5 µg of total RNA	[14, 15, 18,19]
	Real-time RT-PCR	80 pg - 500 pg of total RNA or 6 to 60 copies of mRNA	[15, 16, 20]
	Isothermal amplification (RCA, LAMP, SMART)	10-20 copies of target RNA*	[21, 22, 23, 24, 25, 26, 27]
Enzymatic cleavage	RFLP	0.5-1-µg of cDNA*	[28, 29, 30]
	Ribonuclease Protection Assay	5 femtograms of target RNA or 4,000 to 5,000 copies of mRNA*	[31, 32, 33]
Hybridization	Northern blot	3 - 30 µg of total RNA or 0.01–0.025 fmol of target RNA or 10,000 copies of mRNA	[34, 35]
	FISH	1 RNA molecule/cell	[36, 37,38]
	Microarray	Down to 200ng of total RNA*	[39, 40]
Sequencing	Based on Sanger method: SAGE, CAGE, PMAGE	50 µg of total RNA*	[41, 42, 43, 44]
	High-throughput sequencing: RNA-seq	500 pg of total RNA*	[45, 46, 47]

* requires a step of retro-transcription and amplification to cDNA prior to detection
** Limit of detection may vary according to the target mRNA expression in total RNA

Moreover, in contrast to DNA, the RNA lacks for efficient genotyping technologies since almost all techniques indirectly detect RNA through cDNA. It is important to bear in mind that cDNA analysis can give an absolute abundance of the transcript but is subjected to the stochastic behavior of the transcriptase, which can ultimately corrupt the real concentration of transcripts in the cell, which can be crucial for clinical diagnosis.

1.2 Nanodiagnostics

Nanodiagnostics can be defined as the use and control of nanomaterials combined with biomolecules for molecular diagnostics[53]. In general, the great advantage of working at the nanoscale is that biomolecules and nanostructures are at the same nanometer scale requiring less amount of sample while increasing sensitivity[54,55]. In particular, nanoparticles (NPs) have revealed to be very promising nanostructures for molecular diagnostics due to their unique physicochemical properties such as high surface to volume ratio; enhanced photoemission; high electrical and heat conductivity; and improved surface catalytic activity. These properties may vary depending on their size (e.g. 1-100nm) and composition which are usually inorganic materials[56], such as noble metals (e.g Au, Ag, Pt)[57], magnetic metals (e.g. Fe_30_4, Co)[58,59], semi conductors (e.g. CdSe, CdS)[60,61] or a combinations of the previously mentioned[62,63].
Among these, noble metal NPs are the most promising for the development of diagnostic techniques due to their accessible synthesis and derivatization, and unique size and shape-dependent optical properties[64,65].
For noble metal NPs smaller than the wavelength of the incident light, the interaction between the electromagnetic waves of light and the electrons across the NP's surface induces oscillations of these electrons, giving rise to a phenomenon known as Surface Plasmon Resonance (SPR). This SPR is responsible for the enhancement of scattering and absorption of electromagnetic radiation across the noble metal NPs[66], providing intense colors in the visible spectrum to the colloidal solutions. These optical properties are dependent on the composition, size, morphology, dielectric properties of the surrounding medium and inter particle distances[67,68]. Some of these properties have been explored in the development of new and faster molecular diagnostic techniques, with increased sensitivity and simplicity at lower costs.

1.2.1 Gold Nanoparticles (AuNPs)

Gold nanoparticles (AuNPs) are the most widely reported and promising noble metal NPs for biomedical applications, not only for their well-known syntheses methods, but also for their capacity of bioconjugation[64]. The most common method for AuNPs syntheses is the Turkevich's method, which is based on the reduction of chlorauric acid with sodium citrate[69,70]. The citrate ions act both as reducing and capping agent, enabling a stable and modestly monodisperse colloid aqueous solution of AuNPs that can be easily derivatized with biomolecules usually through a thiol or amine moiety.
Typically, a solution of colloidal gold exhibits a maximum SPR absorption band centered at 520nm with a characteristic red color. Aggregation of the AuNPs leads to a decrease in the inter

particle distance and a concomitant lower resonance frequency, promoting a shift of the SPR absorption peak to longer wavelengths. As a consequence, the colloidal solution changes color from red to blue, a phenomenon that has been explored in the development of colorimetric molecular diagnostic methods[71].

AuNPs have been reported to be successfully bioconjugated with many different biofunctional groups such as antibodies[72,73], carbohydrates[74], peptides[75] and oligonucleotides[76,77,78] towards a specific biomolecular recognition. The derivatization of AuNPs with thiol-modified oligonucleotides (i.e. Au-nanoprobes) has emerged as a promising next generation biomarker technology[54, 65, 79].

Taking advantage of the amazing AuNPs properties, several molecular detection methods with great potential for point-of-care diagnostic have been developed for the specific and sensitive detection of nucleic acids sequences.

The capacity of single stranded nucleic acids (ssDNA) to non-specifically adsorb to the AuNP's surface and increase the stabilization upon salt-induced aggregation has been explored by Li and co-workers[80]. In this colorimetric assay detection is based on non-functionalized AuNPs. The identification of specific sequences within a PCR amplicon is achieved by hybridization with ssDNA probes that would otherwise adsorb to the AuNPs surface and stabilize them. The hybridization of ssDNA probes with the amplicon inhibits their adsorptions to the AuNPs and, consequently, they aggregate upon an increasing ionic strength and the solution changes color from red to blue. If the amplicon has no complementarity to the ssDNA probes, they will remain adsorbed to the AuNPs providing an increased stability to the increasing ionic strength and, thus, the colloidal solution does not aggregate and remains red. This method has been successfully used for the characterization of single nucleotide variations in DNA. Nonetheless, this simple method has some drawbacks, mainly the limited control over the adsorption/desorption of the single stranded oligonucleotides which can give rise to false negatives/positive results[80].

1.2.2 Methods based in Au-nanoprobes

Functionalized AuNPs have been used in a variety of formats towards the development of molecular diagnostics protocols.

Electrochemical detection based on the hybridization between the oligonucleotide target and the Au-nanoprobe has proved to be very sensitive for DNA targets[81]. For example, DNA segments related to the BRCA1 breast-cancer gene have been detected with a detection limit of around 1.2fmol[82]. Still, the electrochemical detection directly on RNA using Au-nanoprobes has not been reported so far.

The colorimetric detection of DNA based on Au-nanoprobes was firstly reported by Mirkin and co-workers in 1996[76]. This approach is known as cross-linking method because it uses two different Au-nanoprobes with complementary sequences to adjacent regions within the target. This way when hybridization of both Au-nanoprobes with the target occurs due to their complementarity, a cross-linked network between AuNPs is formed and the inter particle distance decreases, which leads to a colorimetric change from red to blue. The detection of a single-base mismatch in DNA targets can be successfully reached using this system with appropriate temperature control[70].

Mirkin *et al.* used Au-nanoprobes labeled with Raman-active dyes and oligonucleotides to accomplish multiplexed multicolor detection of DNA and RNA targets reaching a limit of detection of 20fM with a single base resolution[83]. Another example was provided by Glynou *et al.*[84], where the detection of 500 copies of prostate-specific antigen through the hybridization with Au-nanoprobes in a chromatographic stripe (dipstick) can be achieved by naked eye. In this method, similarly to current pregnancy tests, biotinylated RT-PCR products are hybridized to a specific oligo(dA)-tailed probe and loaded on the chromatographic stripe. As the buffer migrates through the stripe, the biotinylated RT-PCR products are immobilized by streptavidin spotted in a specific location of the stripe. Finally, to detect the presence of the DNA target in the streptavidin spot, poly-dT Au-nanoprobes are used to hybridize to the poly-dA probe.

A mRNA expression array was also recently demonstrated by Huber and co-workers[85] by using a surface-immobilized probe to capture the complementary transcripts from total human RNA samples. After hybridization, poly- T(20) Au-nanoprobes hybridized to the -polyA tails of the captured targets. Through this method the authors were able to detect specific transcripts from 0.5µg of human total RNA without enzymatic amplification.

1.2.3 Non-cross-linking method (our method)

A non-cross-linking method for detection of gene expression without the need for retro-transcription was developed by Baptista and co-workers[78]. This approach relies on the differential stability of the Au-nanoprobes in the presence/absence of a complementary target upon increasing ionic strength of the solution. The presence of fully complementary target and its hybridization to the Au-nanoprobe prevents aggregation upon salt addition and the solution remains red. In contrast, the presence of a non-complementary target or a target harboring a single point mismatch cannot prevent the Au-nanoprobe aggregation upon increasing the ionic strength, and the solution changes color from red to blue.

The mechanisms of stabilization by hybridization between target and Au-nanoprobe upon salt addition has been initially proposed to be due to an increase in the electrostatic repulsion

between Au-nanoprobes associated with the negatively charged backbone of the hybridized nucleic acid targets[78,86,87,88]. Recently, steric hindrance has also been thought to be associated to the stabilization in this non-cross-linking method, in which the hybridization to a complementary target provides a greater steric hindrance to the Au-nanoprobe and contributes to prevent inter-particle aggregation[89]. The method has been successfully applied to detected gene expression in 0.3 µg of total RNA without reverse transcription or PCR amplification[78], and *Mycobacterium Tuberculosis* DNA in clinical samples after PCR amplification[86] with a limit of detection of 0.75 µg of total DNA. The method is also able to discriminate single nucleotide polymorphisms in PCR products of relevant biological samples with a sensitivity of 75 fmol/µL[90,91]. More recently, it has been reported the detection and quantification of the BCR-ABL fusion transcript, which is responsible for chronic myeloid leukemia[92]. This detection was reached with discrimination of 50% of complementarity between the target and the Au-nanoprobe, with a limit of detection of 10 ng/µl[92]. This method has also been described to have the ability to differentially detect two distinct target sequences in a multiplex colorimetric assay by using Au-nanoprobes combined with AuAg-alloy nanoprobes, taking advantage of different SPR absorbance peaks[93]. The colorimetric changes that occur in the non-cross-linking method can be assessed directly by the naked eye or quantified through UV-visible spectroscopy [78] or through the use of a light sensitive amorphous/nanocrystalline silicon pi'ii''n photovoltaic sensor developed by the same authors[94]. The colorimetric changes can be quantified through a ratio between the SPR peak of the dispersed solution, around 525nm, and the aggregated SPR peak that shifts to longer wavelengths, usually 15 minutes after salt addition[78,90].

Once the non-cross linking method developed by Baptista and co-workers[78 have proved to have a single base resolution on DNA at room temperature and to have been successfully applied on the direct detection and quantification on mRNA, the work presented in this thesis aim to verify the possibility of the direct detection of single base differences directly on mRNA.

1.3 Proof-of-concept: *BRCA1* targets

The breast cancer 1 (*BRCA1*) gene (MIM 113705), has been widely related to inherited breast and ovarian cancer[95], with many different mutations related to cancer risk variations[96]. *BRCA1* gene is a tumor suppressor gene and the protein product has been associated to numerous nuclear functions such as transcription, recombination, DNA repair and checkpoint control[97]. This 100 kb gene is located on the long arm of chromosome 17 (17q21) and it harbors 24 exons encoding a 1863 amino acid protein[95]. Screening of the complete coding region of *BRCA1* gene is laborious, mainly because of the complex mutation spectrum and the large size of the

gene. Nonetheless, the strong linkage disequilibrium region within this gene enables haplotype based studies. The most frequent SNPs found in *BRCA1* gene are used to characterize these haplotypes[98].

In this work, attention was focused on three of the most frequent SNPs present in *BRCA1* gene, namely, c.2731C>T (dbSNP rs#: rs799917), c.3232A>G (dbSNP rs#: rs16941) and c.3238G>A (dbSNP rs#: rs4986852) – see also Table 1.2.

Table 1.2- Allele frequencies of the most frequent *BRCA1* SNPs

SNP frequency according to HAPMAP-CEU			
	Wild-type	Heterozygous	Homozygous
c.2731C>T	0.434	0.46	0.106
c.3232A>G	0.442	0.451	0.106
c.3238G>A	0.912	0.088	0

All these variations are missense polymorphisms located within exon 11 of *BRCA1*. Nonetheless, their impact on risk of breast and ovarian cancer is not clear. Studies indicate that SNP c.3238G>A may play a role in disease predisposition[99]. The other two SNPs are generally found to be non-pathological[100,101], although some associations with breast cancer have been found[102].

Genetic screening of high risk families for breast cancer is now available in most hospitals from developed countries. Namely, in Portugal this is carried out by the Department of Breast and Ovarian Cancer Risk Evaluation at Instituto Português de Oncologia (IPO). Currently, at IPO, a set of laborious and expensive pre-screening assays towards sample genotyping is required prior to sequencing of the samples of interest. Assessment of gene expression can also be an important tool since it is the deficient protein activity that is thought to be related to disease onset and development. Thus, the analysis of gene expression could help clarification on the involvement of the gene variants in the development of the disease. In fact, RNA studies in inherited breast cancer patients have previously revealed to be an important factor in the survival of chemotherapy[103]. Moreover, deficient RNA expression of *BRCA1* gene has also been related to non-inherited breast cancer[104]. Therefore, the development of Au-nanoprobes for identification of the most frequent SNPs directly in mRNA of patients could help characterize the haplotypes of interest while reducing costs and screening time. By directly targeting mRNA, we might be able to retrieve valuable information regarding which allele is being expressed and, eventually, whether there is any deviation of the standard proportion of either allele expression level. Since the Au-nanoprobe system does not require prior retro-transcription and/or amplification, the eventual enzymatic performance bias would therefore be bypassed.

1.4 Objectives

This work aimed at assessing the possibility of using DNA functionalized AuNPs (i.e. Au-nanoprobes) to detect single nucleotide differences directly in RNA targets via the non-cross-linking method. As proof-of-concept, the three of the most frequent SNPs found in *BRCA1* gene were used as targets.

To achieve this objective, several intermediate objectives were set forward to fulfill the ultimate goal:

- amplicons from patient samples harboring the SNPs of interest were cloned into an expression vector so as to provide plenty of biological material for subsequent tests, to be used as templates for *in vitro/in vivo* transcription reactions and to transform suitable bacterial hosts to create an expression model
- optimization of *in vitro* and *in vivo* expression of the SNP transcripts so as to produce the RNA targets required for method validation and calibration
- synthesis and characterization of AuNPs suitable for functionalization with thiol-modified oligonucleotides
- Au-nanoprobes for the detection of each SNP in RNA were designed and synthesized
- Characterization of the Au-nanoprobes in terms of stability, capacity of hybridization and quantification of a synthetic ssDNA oligonucleotide target, alone and in spike-in mixtures of total RNA purified from suitable cells
- Evaluation of the Au-nanoprobes detection capability in complex mixtures, mimicking real biological samples using *in vitro* transcription products alone and in spiked mixtures
- Assessment of SNP discrimination within target RNA molecules.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment and specialized material

- UV-Vis Spectrophotometer UV Mini-1240 (Shimadzu, Germany),

- UV-Vis Spectrophotometer Nanodrop ND-1000 (Nanodrop Technologies, USA)

- Microplate reader Infinite M200 with Absorbance module (Tecan, Switzerland)

- Thermal Cycler DNA Engine (Bio-Rad, USA), Thermal Cycler Tgradient (Biometra, Germany)

- Gel Doc XR+ Molecular Imager system (Bio-Rad, USA)

- Ultrasonic bath Elmasonic S10H (Elma, Germany)

- E-Gel® iBase™ Power System with E-Gel® Safe Imager and E-Gel® EX pre-cast gel, 2% (Invitrogen, USA)

- pH meter Basic 20 with combined glass electrode 5209 (Crison, Spain)

- Quartz absorption cells – 105.202-QS (Hellma, Germany)

- 384 well small volume, LoBase Polystyrene microplates, black – Cat.No.788096 (Greiner Bio-One)

- NAP-5 columns (GE Healthcare, Sweden)

- Costar® Spin-X® Centrifuge Tube Filters, 0.22 µm Pore CA Membrane Sterile (Corning, USA)

- Spectra/Por 1 Dialysis Tubing, 10mm width, part no. 132645 (Spectrum Labs, USA)

2.1.2 Chemical Reagents

Table 2.1 - Chemical reagents

Reagent	CAS number	Distributer
Agarose	9012-36-6	Invitrogen
Chloroform	67-66-3	Merck
Glacial acetic acid	64-19-7	
Magnesium chloride	7786-30-3	
Potassium chloride	744-40-7	
Sodium chloride	7647-14-5	
Sodium hydroxide	1310-73-2	
Sodium phosphate dibasic dihydrate	10028-24-7	
Sodium phosphate monobasic monohydrate	10049-21-5	

Reagent (cont.)	CAS number	Distributer
Ampicillin	69-53-4	Sigma-Aldrich
Boric acid	10043-35-3	
Glycerol	56-81-5	
Glucose	50-99-07	
Gold(III) chloride trihydrate	16961-25-4	
Manganese chloride	13446-34-9	
Methyl sulfoxide (DMSO)	67-68-5	
PIPES	5625-37-6	
Potassium hydroxide	1310-58-3	
Sodium citrate tribasic dihydrate	6132-04-3	
Sodium Dodecyl Sulfate (SDS)	151-21-3	
Ethylenediamine tetraacetic acid	60-00-4	Fluka
Dithiothreitol (DTT) solution, 1M	3483-12-3	
Phenol	108-95-2	
Tris(hydroxymethyl)aminomethane	77-86-1	
Diethyl Pyrocarbonate	4525-33-1	
Tryptone	91079-40-2	Difco
Bacteriological agar	9002-18-0	
Proteinase K	39450-01-6	Roche
Yeast extract	8013-01-2	USB
Gel red	n.a	Biotium
Calcium chloride	10035-04-8	AppliChem
Bacteriological agar	9002-18-0	Difco
Peptone	n.a	Panreac
Sodium acetate	6131-90-4	Jose M. VAz Pereira, LDA

2.1.3 Solutions

10mM (Sodium) Phosphate buffer, (pH8)

9.32 mM Na_2HPO_4
0.68 mM NaH_2PO_4

Synthesis Buffer I

2% (w/v) SDS
10 mM Phosphate buffer, pH 8
Sterilize by filtration and store at 4ºC.

Synthesis Buffer II	1.5 M NaCl 0.01% (w/v) SDS 10 mM Phosphate buffer, pH 8 Sterilize by filtration and store at 4ºC
AL I .	50 mM Glucose 25 mM Tris-HCl, pH 8 10 mM EDTA, pH 8 Sterilize by autoclaving and store at 4ºC.
AL II	200 mM NaOH 1% (w/v) SDS Prepare fresh before use (room temperature).
AL III	3 M Sodium acetate (adjust pH to 4.8 with glacial acetic acid) Store at 4ºC.
Luria-Bertani medium (LB medium)	1% (w/v) tryptone 0.5% (w/v) yeast extract 171 mM NaCl Adjust to pH 7 with NaOH. Sterilize by autoclaving and store at 4ºC. (For solid medium, add 1.4% (w/v) of Agar)
Super Optimal Broth (SOB medium)	2% (w/v) tryptone 0.5% (w/v) yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ * 10mM $MgSO_4$* Adjust to pH 7 with NaOH. Sterilize by autoclaving and store at 4ºC. *Add sterile $MgCl_2$ and $MgSO_4$ just before use.
Super Optimal broth with Catabolite repression (SOC medium)	Same as SOB medium plus add 20 mM glucose (sterile) after autoclaving SOB medium and cooling down to 60ºC or less.
Yeast Extract Peptone Dextrose medium (YPD medium)	1% (w/v) yeast extract 2% (w/v) Peptone 2% (w/v) Glucose Sterilize by autoclaving and store at 4ºC.
Terrific Broth medium (TB medium)	10 mM PIPES 15 mM $CaCl_2$ 250 mM KCl 55 mM $MnCl_2$ Adjust pH to 6.7 with KOH or HCl. Sterilize by filtration and store at 4ºC.

2.1.4 Biological Material

2.1.4.1 Molecular Biology

Enzymes

DreamTaq DNA polymerase (Fermentas, Canada)

T7 RNA polymerase (Fermentas, Canada)

Restriction enzyme; *XhoI* and *AluI* (New England Biolabs, USA)

DNAse I, RNase-free (Fermentas, Canada)\

Lyticase (Sigma-Aldrich,USA)

Lysozyme (Sigma-Aldrich,USA)

DNA size markers

GeneRuler™ DNA Ladder Mix, ready-to-use (Fermentas, Canada)

RiboRuler™ High Range RNA Ladder (Fermentas, Canada)

Kits

CloneJET™ PCR Cloning Kit (Fermentas, Canada)

GeneJET™ RNA purification Kit (Fermentas, Canada)

Quant-iT™ OliGreen® ssDNA Assay Kit (Invitrogen, USA)

Microorganisms

Escherichia coli DH5α

Escherichia coli BL21 (DE3) pLysE (Bioline, USA)

Saccharomyces cerevisiae CBS 8803

2.1.4.2 Oligonucleotides

All ssDNA oligonucleotides were acquired from STAB VIDA, Lda.

Table 2.2 - Primers

Designation	Sequence (5' > 3')	Tm (ºC)	Observation
pJET1.2_Fw	CGACTCACTATAGGGAGAGCGGC	60.4	pJET1.2 primers
pJET1.2_Rev	AAGAACATCGATTTTCCATGGCAG	55.8	
pJET1_Fw	GCCTGAACACCATATCCATCC	51.9	For amplification of phage T7 promoter sequence
BC1_2731_Fw	TGGTACTGATTATGGCACTCAGGAAAGTAT	59.6	Amplicon harboring SNP c.2731 (C/T)
BC1_2731_Rev	GTGACTTTTGGACTTTGTTTCTTTAAGGACC	59.5	
BC1_3232/38_Fw	GGCAACGAAACTGGACTCATTACTCCA	59.8	Amplicon harboring SNP c.3232(A/G) and SNP c.3238(G/A)
BC1_3232/38_Rev	GTTGCAAAACCCCTAATCTAAGCATAGCA	60.2	

Table 2.3 - Synthetic ssDNA targets

Designation	Sequence (5' > 3')
sBC1_2731C	TTCAAAGCGCCAGTCATTTGCTCCGTTTTCAAATCCAGGAAATGCA
sBC1_2731T	TTCAAAGCGCCAGTCATTTGCTCTGTTTTCAAATCCAGGAAATGCA
sBC1_3232_38AG	ACATTAGAGAAAATGTTTTTAAAGAAGCCAGCTCAAGCAATATTAATGAAGTAGGTT
sBC1_3232_38GA	ACATTAGAGAAAATGTTTTTAAAGGAGCCAACTCAAGCAATATTAATGAAGTAGGTT

Table 2.4 - Thiol-modified ssDNA probes

Designation	Sequence (5' > 3')	Modification
BC1_2731C	TGCATTTCCTGGATTTGAAAACG	5'-thiol
BC1_2731T	TGCATTTCCTGGATTTGAAAACA	5'-thiol
BC1_3232A	TCTTTAAAAACATTTTCTCTAATGT	3'-thiol
BC1_3232G	CCTTTAAAAACATTTTCTCTAATGT	3'-thiol
BC1_3238G	AACCTACTTCATTAATATTGCTTGAGC	5'-thiol
BC1_3238A	AACCTACTTCATTAATATTGCTTGAGT	5'-thiol

Note: Thiol-modified ssDNA were resuspended in 100 µL of 1 M DTT and incubated for 1 h at room temperature followed by the addition of 900 µL of sterile milli-Q water to achieve a final concentration of 0.1M DTT.

2.1.4.3 Human genomic DNA samples

Human genomic DNA samples harboring SNPs in the *BRCA1* gene were gently provided by Drª. Fátima Vaz from the Molecular Biology Department and Breast Cancer Risk Evaluation Clinic, Instituto Português de Oncologia de Lisboa, Francisco Gentil.

Table 2.5 - Biological samples genotype

SNP in *BRCA1* gene	c.2731 (C/T) p.P871L rs799917		c.3232(A/G) p.E1038G rs16941		c.3238 (G/A) p.S1040N rs4986852	
Genotype	CC	TT	AA	GG	GG	GA

2.2 Methods

2.2.1 Target Sequence Preparation

2.2.1.1 Preparation of competent *E. coli* cells (adapted from Inoue *et al.*[105])

1. *E. coli* DH5α cells were inoculated on a LB agar plate and incubated at 37ºC overnight.
2. A large colony was inoculated in 125 mL SOB medium in a 500 mL flask and grown at 20ºC with vigorous shaking up to OD_{600nm} = 0.5 (normally 24 - 36 hours).
3. The flask was placed on ice for 10 min.
4. Cells were pelleted by spinning at 1,663× g for 10 min at 4ºC.
5. The cells were gently resuspended in 40 mL ice-cold TB and stored on ice for 10 min.
6. Cells were pelleted by spinning at 1,663× g for 10 min at 4ºC.
7. The cells were gently resuspended in 5 mL ice-cold TB and 350 µL DMSO (DMSO was stored at -20ºC o/n before use).
8. Competent cells were aliquoted in 200 µL fractions and stored at -80ºC until use.

2.2.1.2 PCR amplification

Reaction Mixture

Component	**per reaction (V_{total}=20µL)**
10X DreamTaq buffer inc. $MgCl_2$	2 µL
dNTP mix - 5 mM each	0.8 µL
Forward primer - 10 µM	0.5 µL
Reverse primer - 10 µM	0.5 µL
Template DNA	100 ng

DreamTaq DNA polymerase - 5u/µL	0.1 µL
Water, nuclease-free	to 20 µL

Reaction program

A first target denaturation was performed at 94ºC for 2 min, followed by 25 cycles of PCR, each cycle consisting of 95ºC for 30 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 45 seconds and with a final extension step at 72ºC for 7 min.

These conditions were used for all PCR amplifications. Amplicons' size and integrity were confirmed by electrophoresis (90 V, 1 hour) in 1% agarose gel with 1x TBE. The expected size of the amplicons can be found in Appendix A.

2.2.1.3 Cloning

2.2.1.3.1 Ligation

1. The amplicons of interest were extracted using the E-Gel® electrophoresis system with E-Gel® EX pre-cast 2% agarose gels, following the manufacturer's instructions.
2. The extracted amplicon (100 ng) was inserted into a linearized blunt-end pJET1.2 cloning vector using the GeneJET™ PCR Cloning Kit and following the manufacturer's Sticky-End protocol.

2.2.1.3.2 Transformation of *E. coli* DH5α

1. The ligation product (2 µL) was added to 20 µL of E. coli DH5α competent cells and let to rest on ice for 30 minutes. 150 ng of purified pUC18 plasmid was used as positive control and as negative control, the ligation product was replaced by sterile H_2O.
2. The cells were then submitted to a heat shock at 42ºC for 90 seconds and rapidly transferred to ice.
3. After 2 minutes on ice, 80 µL of SOC medium was added and the cells were incubated for 1 hour in a shaking water bath at 37ºC.
4. After incubation, 100 µL of the transformed competent cells were cultured on LB agar plates with 100 µg/mL ampicillin and incubated o/n at 37ºC.
5. Up to three transformed colonies were resuspended in 25 µL of sterile H_2O.
6. Two microliters of transformed cells were used to perform PCR using pJET1.2 Fw and a specific reverse primer to each inserted amplicon. The remaining cells were inoculated in 2 mL of LB medium with 100 µg/mL ampicillin and incubated o/n in a shaking water bath at 37ºC.

7. Stock solutions of the transformed cells were prepared by adding 300 µL of glycerol to 700 µL of culture and stored at -80ºC. The remaining volume of culture was used to extract and purify the cloned plasmid, following step 2. onwards of the "Plasmid extraction and purification" protocol.

2.2.1.3.3 Transformation of *E. coli* BL21 (*DE3)* pLysE

E. coli BL21 (*DE3)* pLysE competent cells (100 µl) were transformed with 10 ng of plasmid DNA (pBC1_2731C, pBC1_2731T, pBC1_3232_38AA and pBC1_3232_38GG) purified from the DH5α clones following manufacture's transformation protocol. As positive control 200 pg of pUC19 plasmid provided by the manufactures were used.

2.2.1.4 Plasmid extraction and purification

1. An *E. coli* colony was inoculated in 2 mL of LB medium and 100 µg/mL ampicillin and incubated o/n in a shaking water bath at 37ºC.
2. Cells were pelleted by spinning at 16,707× g for 2 min at 4ºC in 1.5 mL eppendorfs.
3. The supernatant was discarded and the pellet was resuspended in 100 µL of ice-cold AL I solution.
4. After 5 minutes on ice, 200 µL of AL II solution was added and mixed by inversion.
5. After 5 minutes on ice, 150 µL of AL III solution was added and vigorously mixed by vortex.
6. After 5 minutes on ice, the lysate was centrifuged at 21,460× g for 5 minutes at 0ºC.
7. The supernatant was transferred to a sterile 1.5 mL eppendorf and 2 volumes of ice-cold absolute ethanol and 0.3 M of sodium acetate were added.
8. The plasmid DNA was left to precipitate at -20ºC o/n (or at -80ºC for 2 hours).
9. Afterwards, the precipitate was centrifuged at 21,460× g for 15 minutes at 0ºC and the supernatant was discarded.
10. The pellet was washed with 500 µL of ice-cold 70% ethanol and centrifuged at 21,460× g for 5 minutes at 0ºC.
11. The supernatant was discarded and the pellet was dried using a speed-vac system and resuspended in 50 µL of sterile H_2O.
12. RNase A was added to a final concentration of 25 µg/ml and incubated for 1 hour at 37ºC.
13. Two extractions with 1 volume of phenol were performed, followed by one extraction with 1 volume of chloroform.
14. To precipitate the plasmid DNA, the procedures from step 7 to 11 were repeated.

2.2.1.5 Purification of template for *in vitro* transcription

The plasmids harboring the sequences of interest were used as template for PCR using the pJET1Fw primer and the reverse primer specific to each insert. The use of pJET1Fw primer allows the amplification of the phage T7 promoter sequence within the amplicon. The PCR amplification was carried out as described in **2.2.1.4 PCR amplification**. The resulting amplicons were purified by phenol/chloroform extraction, followed by precipitation as described in **2.2.1.3 Plasmid extraction and purification, step 13 and 14.** The pellet was then resuspended in 50µL of DEPC treated water.

2.2.1.6 *In vitro* Transcription

Reaction Mixture

Component	**per reaction (V_{total}=50µL)**
15X Transcription buffer	10 µL
NTP mix – 10mM each	10 µL
Linear template DNA	1 µg
T7 RNA polymerase	1.5 µL
DEPC- treated water	to 50 µL

Reaction program

The reaction mixtures were incubated at 37°C for 2 hours, followed by heat inactivation of the enzyme at 70ºC for 10 minutes.

2.2.2.1.6.1. *In vitro* transcript purification

A. Purification from agarose gel

1. The *in vitro* transcript was purified by excision from 2% agarose gel after electrophoresis (40 V, 2h30, 1x TAE).

A.I - Squeeze and freeze

1. The excised gel slice was frozen at -80°C during 20 minutes followed by centrifugation in a Spin-X tube according to the manufacturer's instructions. The transcript was collected in microcentrifuge tube while the agarose is retained on the Spin-X membrane.

2. Afterwards, the transcript was precipitated and washed as described in **2.2.1.3 plasmid extraction and purification**, step 7 to 11.

The concentration and quality of the purified *in vitro* transcripts were determined by UV/Vis spectroscopy at 260nm and by the 260nm/230nm and 260nm/280nm ratios. The correct size and integrity of the transcript was verified by agarose gel (2%, 1x TBE) electrophoresis (90V, 1h30).

A.II - Electroelution in dialysis tubing

Preparation of Dialysis Tubing

1. Tubing were cut into pieces of 10 cm length and left to boil for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1mM EDTA (pH 8) treated with DEPC 0.1% (v/v).
2. Dialysis Tubing were then thoroughly rinsed with water treated with DEPC and left to boiled 10min in 1mM EDTA (pH 8)
3. Tubing were allowed to cool and stored at 4ºC until further use.

Electroelution

1. One end of the bag was sealed with plastic clip, and the tubing was filled with 1x TAE buffer.
2. The band on interest was excised from the gel (as mentioned in *Purification of the gel* step 1) and placed inside the tubing.
3. The gel slice was allowed to sink to the bottom of the bag and the buffer was squeezed out leaving just enough to maintain the slice in contact with the buffer, and the bag was then sealed with another plastic clip.
4. The bag was immersed in an electrophoresis tank, maintaining the gel slice in parallel with the electrodes. Electric current (30V) was applied for 40 minutes.
5. Afterwards polarity was reversed for 1 minute to release any RNA that could adhere to the tubing.
6. The bag was then opened and the TAE recovered, precipitated and washed as described in **2.2.1.3 plasmid extraction and purification, step 7 to 11**.

B. Desalting NAP-5 column

1. Purification of the *in vitro* transcript products through a desalting NAP-5 column, according to manufacturer's instructions, using 500 µl of water treated with DEPC as eluent.

C. Phenol/chloroform extraction

1. Two extractions with 1 volume of phenol, followed by one extraction with 1 volume of chloroform were performed and left to precipitate with two volumes of absolute ethanol and 0.3 M of sodium acetate at -20ºC o/n (or at -80ºC for 2 hours).

Note: For the NAP-5 desalting and phenol/chloroform extraction, elimination of the template was performed with DNAse I as described by the manufacture (2U of DNAse I per 50 µL of transcription reaction, at 37 ºC for 15 min).

2.2.1.6.2 Target Quantification

Quantification of synthetic ssDNA targets

All synthetic ssDNA targets were quantified through UV-vis spectroscopy using the extinction coefficient at 260nm of each ssDNA oligonucleotides, calculated according to OligoCalc bioinformatic tool available on-line [106].

Quantification of *in vitro* transcripts

Quantification of the *in vitro* transcripts was performed by agarose gel (2%, 1x TBE) electrophoresis (90 V, 1h30) analysis, using the fluorescence intensity of a known DNA/RNA ladder as reference. For RNA quantification the comparison of intensity was made using the 500 b fragment of the RNA ladder, RiboRuler™High Range, as reference. For the DNA template quantification, the 500pb fragment of the GeneRuler™ DNA Ladder Mix was used as fluorescent reference. The DNA template concentration within the purified transcripts was used to ensure that concentration of template does not exceeded the limits of detection reported (900ng) [90,91].

2.2.1.7 Total RNA Extraction from *S. cerevisiae*

An *S. cerevisiae* colony was inoculated in 400ml YPD medium and incubated o/n in a shaking water bath (150 rpm) at 25ºC. Yeast cells were harvested at the exponential phase of growth (OD_{600nm} = 0.5 ~ 1). Total yeast RNA purification was performed using GeneJET™ RNA Purification as described in the manufacture's protocol. Integrity of total RNA was verified by agarose gel (2%, 1x TBE) electrophoresis (90V, 1h30). The concentration and quality of the transcripts was determined by UV/Vis spectroscopy at 260nm and by 260nm/230nm and 260nm/280nm ratios, respectively.

2.2.1.8 IPTG induction of *E. coli* BL21 and total RNA extraction

1. *E.coli* BL21 (DE3) pLysE clones harboring the plasmid pBC1_3232A_38A or pUC19 were inoculated in 100 mL of LB broth containing 50µg/ml of chloramphenicol and 100 µg/mL ampicillin and incubated o/n in a shaking water bath at 37ºC until stationary phase was reached.

2. Afterwards, a dilution with new LB broth (provided with the antibiotics mention in step 1) was performed to achieve 0.05-0.1 OD_{600}. These cultures were incubated at 37°C until OD_{600}=0.4 (~ 3 hours).
3. Each flask was split in two: one for induction with 1mM IPTG and the other with no addition of IPTG, for expression control, and cultures were let to shake for 2 hours at 37ºC.
4. Total *E.coli* RNA extraction was performed by using GeneJET™ RNA Purification as described in the manufacture's protocol. Integrity of total RNA was verified by agarose gel (2%, 1x TBE) electrophoresis (90V, 1h30). The concentration and quality of the transcripts was determined by UV/Vis spectroscopy at 260nm and by the ratio 260nm/230nm and 260nm/280nm.

2.2.2 Nanoprobes Preparation

2.2.2.1 AuNPs synthesis (adapted from Lee and Meisel[107])

All glass material used in the synthesis process was previously treated with *aqua regia* (1 vol HCL: 3 vol HNO_3) and washed with ultrapure milli-Q H_2O (18.2 MOhm.cm resistivity at 25 °C).

1. In a 500 mL round bottom flask, 250 mL of 1 mM $HAuCl_4$ were brought to a boil while vigorously stirring.
2. While in reflux, 25 mL of 38.8 mM sodium citrate were quickly added and the mixture was kept refluxing for 15 minutes with continuous stirring.
3. The colloidal solution was left to cool to room temperature while keeping the continuous stirring.
4. The colloidal solution was then transferred to a 250 mL Erlenmeyer flask with a ground glass cap and stored in the dark at room temperature until further use.
5. AuNPs 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}.
6. Morphological characterization of the AuNP was performed by Transmission Electron Microscopy (TEM).

2.2.2.2 TEM analysis

Samples of AuNPs were sent to Instituto de Ciência e Engenharia de Materiais e Superfícies (ICEMS/IST), Portugal, for TEM analysis. The samples were prepared by depositing 10 µL of the as-prepared colloidal solution in carbon copper grids, washing twice with 10 µL of Milli-Q water, and air drying the samples afterwards. TEM was performed with a HITACHI H-8100

microscope operated at 200 kV. Particle size and polydispersity were determined from the TEM pictures using the imaging software Carnoy 2.0, by analyzing at least 100 AuNPs.

2.2.2.3 Nanoprobe synthesis - Ultrasound method (adapted from Hurst *et al.*[108])

1. Three extractions with two volumes of ethyl acetate to 500 µL of thiol-modified oligonucleotide were performed.
2. The aqueous phase was further purified through a desalting NAP-5 column, according to manufacturer's instructions, using 10 mM phosphate buffer (pH8) as eluent.
3. The purified thiol-modified oligonucleotides were quantified by UV/Vis spectroscopy using the extinction coefficient at 260 nm provided by the manufacturer. Purified thiol-modified oligonucleotides were added to the colloidal solution of AuNPs in 1:200 (AuNP:oligos) ratio.
4. Synthesis Buffer I was then added to achieve a final concentration of 10 mM phosphate buffer (pH8), 0.01% (w/v) SDS. The solution vial was then submersed in an ultrasound bath for 10 seconds and let to rest at room temperature for 20 minutes.
5. Afterwards, a certain volume of Synthesis Buffer II was added to sequentially increase ionic strength up the final concentration of 0.05, 0.1, 0.2 and 0.3 M NaCl, respectively. Aftereach buffer addition, the vial was submersed in an ultrasound bath for 10 seconds and let to rest at room temperature for 20 minutes before the next increment.
6. After an o/n period at room temperature, the functionalized AuNPs were distributed in 1.5 mL eppendorfs and centrifuged at 21,460× g for 20 minutes.
7. The supernatant was discarded and the resulting oily pellet was washed twice with 1 mL of 10 mM phosphate buffer (pH8) and once with 1 mL of PBS solution
8. The supernatant was then finally redispersed in 500 µL/eppendorf of the same PBS solution. The resulting solutions of each eppendorf were then gathered in a polypropylene 25 mL vial with a conical skirted base and stored in the dark at 4ºC until further use.
9. The final concentration of functionalized AuNPs (i.e. Au-nanoprobes) was determined by the Lambert–Beer law assuming a molar absorptivity for the plasmon resonance band maximum (526 nm) of 2.33×10^{8} M^{-1} cm^{-1}.
10. Au-nanoprobes aliquots (13 nM) were prepared from stock solution using PBS as eluent and stored in the dark at 4ºC until further use.

2.2.2.4 Au-nanoprobes stability assays

1. Solutions were prepared according to the following table 2.6.

Table 2.6 - Stability assays

Component	Stock Concentration	Final concentration*
Au-nanoprobe	13 nM	2.5 nM
$MgCl_2$	0.3 M	0, 20, 30, 40, 50, 60 and 70 mM
or		
NaCl	4 M	0, 0.1, 0.5, 1, 1.5, 2 and 2.5 M

*10 mM phosphate buffer was added to fulfill a final volume of 30 µL

2. The solutions were heated for 10 minutes at 95ºC and let to cool for 30 minutes at room temperature.
3. Salt was added to the solutions and UV–visible spectrum were registered 15 minutes after salt addition.

2.2.2.5 Non-cross-linking hybridization assay

1. Assay solutions were prepared by mixing the Au-nanoprobe solution (final concentration of 2.5 nM) to the appropriate target (final concentration varied from 0.03 pmol/µL to 1 pmol/µL), and using 10 mM phosphate buffer to fulfill the final volume (60 µL for spectrophotometer measurements or 30 µL for microplate measurements). As targets: the complementary target is fully complementary to the probe (detecting the allele of interest), the mismatch target has only one base of difference (i.e it mimics the non-complementary allele for the same locus). As negative control a non-complementary target was added to solution, i.e a target from other locus than the one tested in the assay. A blank solution was prepared replacing DNA/RNA targets with 10mM phosphate buffer.
2. The solutions were heated for 10 min at 95ºC and then allowed to cool down for 30 minutes at room temperature.
3. $MgCl_2$ was added to the final concentration determined by the Au-nanoprobe stability assays. UV-visible spectrum were registered 15 minutes after salt addition.

Spike-in assay

Assays were performed with 2.5 nM of Au-nanoprobes in a final volume of 30 µL. Fixed concentrations of the ssDNA oligonucleotide target (complementary or non-complementary target, as described in **2.2.2.5 Non-cross-linking hybridization assay step 1**, were added to a final concentration of 120 fmol/µL (1.6 ng/µL). Total RNA of *S.cerevisiae* was added to a final concentration of 1.6, 160, 228.6, 400, 640 ng/µL, in order to have decreasing % of ssDNA synthetic oligonucleotide target within the assays. To fulfill the final volume, 10mM phosphate

buffer (pH8) was added. A blank assay was performed by adding only *S.cerevisiae*'s total RNA. As negative control an assay was performed with a non-complementary target.

2.2.2.6 Determination of Au-nanoprobes' density by fluorescent assays (adapted from Sato *et al.* [109])

1. To release the thiol-oligonucleotides from the AuNPs surface, DTT was added (final concentration 100 mM) to 2.5 nM Au-nanoprobes in a total volume of 100 µL 1x TE.
2. Standard solutions were prepared by sequentially diluting thiol-modified oligonucleotides harboring the same sequence as the probes (concentration range: 7.81 to 250 nM), submitting these solutions to the same conditions as Au-nanoprobes' samples, as described in step 1.
3. Following 48 h at room temperature, both standard and sample solutions were centrifuged at 21,460× g for 20 minutes and 50 µL supernatant were used to quantify the ssDNA in solution by using the Quant-iT™ OliGreen® ssDNA Assay kit according to the manufacturer instructions.

3. RESULTS AND DISCUSSION

3.1. Biological Sample preparation

Human genomic DNA samples harboring SNPs in the *BRCA1* gene were gently provided by Drª. Fátima Vaz from Instituto Português de Oncologia de Lisboa, Francisco Gentil (Table 2.5 **2.1.4.6 Human genomic DNA samples**). Based on these variants a set of synthetic oligonucleotides were designed to harbor the sequence variants of interest and used to test the capacity of Au-nanoprobes hybridization to the complementary targets (sBC1_2731C, sBC1_2731T, sBC1_3232_38AG and sBC1_3232_38GA). Simultaneously to achieve the *in vitro/in vivo* transcripts needed for the proof-of-concept, human genomic DNA samples harboring the SNP of interest were cloned into a pJET1.2 vector and subsequently transformed in *E. coli* DH5α, to guarantee a more stable insert and higher plasmid yield, and in *E. coli* BL21(DE3) pLysE, for *in vivo* transcription. The pJET1.2 vector harbors a T7 promoter sequence allowing *in vitro* or *in vivo* transcription of the insert sequence (Appendix B - Figure A.1). After screening of the correct orientation of the insert (by PCR screening) four different clones were sequenced (Appendix C): two clones harboring a 381 bp sequence with the c.2731C or c.2731T allele (pBC1_2731C and pBC1_2731T, respectively); and two clones harboring a 394 bp sequence with the c.3232A and c.3238A or c.3232G and c.3238G alleles (pBC1_3232_38AA and pBC1_3232_38GG, respectively).

3.1.2 *In vitro* transcription

An *in vitro* transcription was carried out using amplicons as template, which were obtained by PCR amplification of the cloned fragments, as described by the manufactures. The transcripts' integrity was confirmed by agarose gel electrophoresis for which the plasmids pBC1_2731C/T provide a transcript with 431 bases (b) and the plasmids pBC1_3232_38AA/GG a transcript with 444 b. Figure 3.1 shows the products of transcription, obtained from the pBC1_2731C, where the transcript (lower molecular weight) can be observed along with the DNA template and another product, a hybrid DNA/RNA.

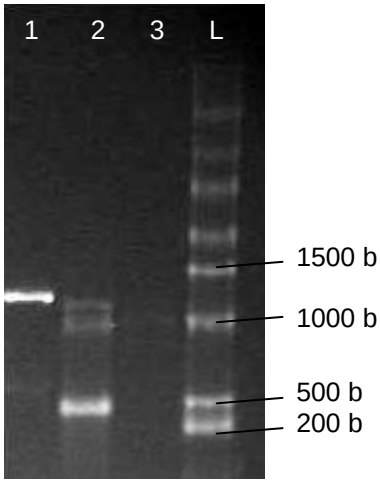

Figure 3.1- *In vitro* transcription. Agarose gel (2%, 1x TBE, 0.5x Gelred ™) electrophoresis (80 V, 1h30). 1) Template BC1_2731C for transcription (473 bp); 2) *in vitro* transcription (resulting transcript of 431b) using BC1_2731C as template; 3) *in vitro* transcription using BC1_2731C as template, after incubation with DNAseI; L) RiboRuler™High Range RNA Ladder.

The *in vitro* transcript cannot be directly used in detection assays because it induces aggregation of Au-nanoprobes prior to the hybridization step (**3.3.2 *in vitro* RNA transcript**). This issue has been previously described and the aggregation was suggested to be induced by the components within the transcription buffer[110]. Additionally, the presence of the template may also interfere with transcript quantification and probe stabilization. For these reasons the *in vitro* transcripts needed to be purified prior to their use in the non-cross-linking assay. This task revealed to be the most difficult one in the development of this work.

The extraction and purification of the transcript from agarose gel electrophoresis is a simply way to ensure only the presence of the RNA molecules of interest and, therefore, have a greater control over the assays. However to avoid contaminations or transcript damage, only a part of the agarose gels containing samples for slicing reference were stained with the fluorescent dye and submitted to UV light. The remaining gel was free from dye and UV exposure and the samples were sliced using the stained part of the gel as reference. Afterwards, purification of the transcript products within the slice of gel was carried out by two different methods:

i) squeeze and freeze method using a Spin-X tube. In this method the gel slice is frozen and then eluted through a column by centrifugation, retaining the agarose and the transcript and buffer are collected. This method allowed to isolate RNA (Appendix D, Figure A.2), but with a very low yield not sufficient to be used in the non-cross-linking assays.

ii) membrane dialysis. The possibility that a significant amount of transcript could remain in the agarose fraction or in the column's membrane, leaded to try a purification using membrane dialysis. This method revealed to have even less yield and, therefore, there was a need to change the purification strategy.

Since the transcription buffer strongly induces nanoprobe aggregation, thus being the main impeditive cause for carrying out the assays, the next step was to purify the transcription product. Two different approaches were made in parallel.

iii) NAP-5 column, a desalting column. A simple elution of the *in vitro* transcription product through a NAP-5 column had been effective for other transcription products purification used for detection by the non-cross-linking method [110]. Unfortunately, in this case, the purified product induced stabilization of the Au-nanoprobes in the negative control. These results will be discussed later (see **3.3 *in vitro* RNA transcript detection**).

iv) phenol/chloroform extraction. T7 RNase manufacturers' suggest a phenol/chloroform extraction for the purification of the transcript and removal of the polymerase enzyme. This method revealed not to interfere with the capability of detection of the Au-nanoprobe system (see **3.3 *in vitro* RNA transcript detection**).

These two last purification strategies do not manage to eliminate the DNA template from the purified solution. For this reason, an incubation with DNAse I was also performed. As a result, the DNA template and DNA/RNA hybrids were digested, but hydrolysis of RNA alone was also observed as it can be observed in Figure 3.1. This may be due to contamination of RNases in the DNase I. In fact residual RNase activity in DNase preparations have been already reported [111]. Considering this, the colorimetric assays with *in vitro* transcripts were performed without digesting the samples with DNase I and a parallel assay with the template alone was also performed to rule out the possible interference of the DNA template (see **3.3 *In vitro* transcript**).

3.2 Synthesis and Characterization of Au-nanoprobes

Synthesis of AuNPs

Gold nanoparticles were synthesized following the citrate reduction method[107]. The resulting colloid suspension of AuNPs was then characterized by UV/Vis spectroscopy and transmission electron microscopy (TEM) (Figure 3.2A).

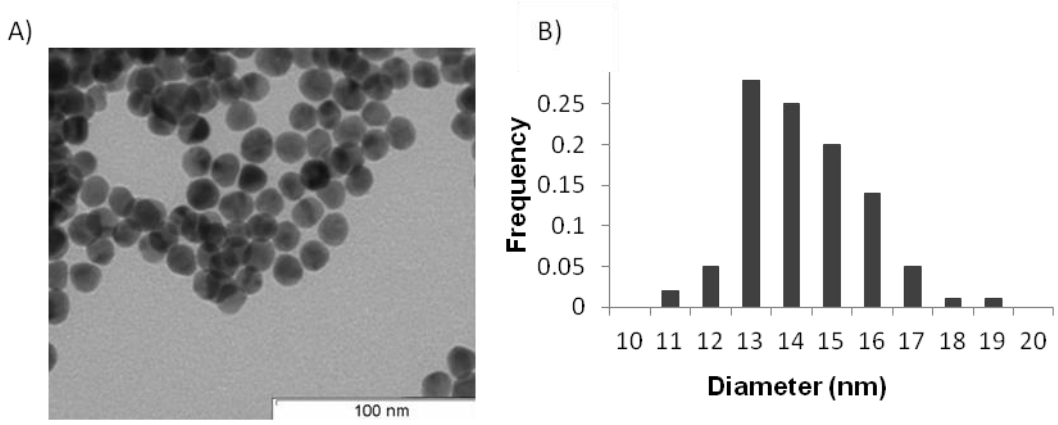

Figure 3.2 - AuNPs characterization. (A) TEM image of AuNPs. **(B)** Size histogram corresponding to the measurements of 120 AuNPs. AuNPs diameter (nm)= 14.2±1.5 (Average±STD)

AuNPs showed an average diameter of circa 14 nm, as determined by the measuring of 120 NPs from 5 TEM micrographs (Figure 3.2B), in agreement with previous reports following the same method[90,91,92].

Probe Design

To study the single base mismatch resolution of the non-cross-linking method in RNA transcripts, three different SNPs located in the *BRCA1* gene coding region were used as proof-of-concept. For each SNP two probes were designed in order to detect each allele. The probes were derived from the complete coding sequence of *BRCA1* (GenBank accession no. U14680). In order to hybridize to the RNA, the thiol-modified oligonucleotides were designed to be the antisense sequence, so as to be complementary to the RNA target.

The probes harbored between 23 to 27 nucleotide sequences and were designed such as the polymorphism is located in the position further from the AuNP, hence the opposite end from the thiol moiety, in order to maximize availability to hybridization as previously described by Doria *et al*. [88]. For this reason all probes were designed in order to have the SNP site recognition in the opposite end of the thiol moiety that was located at the 5'end for all probes, except for two probes: BC1_3232A and BC1_3232G, that have the thiol moiety at the 3´end. These two last probes were designed with a 3'- thiol moiety so as to recognize the SNP c.3232A>G and avoid the superposition of the probe sequence with the nearby SNP c.3238G>A. This way, an independent discrimination between the two different SNPs using four different probes (i.e. BC13232A/G and BC1_3238G/A) may be attained (Figure 3.3).

Figure 3.3 - Complementarity between probes and targets. The BC1_3232A probe, with the variant nucleotide in the 5´end of the modified oligonucleotide, and BC1_3238G probe, with the variant nucleotide in the 3' end of the modified oligonucleotide, both hybridize to the synthetic oligonucleotide sBC1_3232_38AG.

The AuNPs functionalization was performed according to the ultrasound method described by Hurst *et al*.[108] and using a ratio of 200 oligonucleotides per AuNP. This method is preferred over the so called classic method described by Storfhoff *et al*.[70] since it has been reported to allow better reproducibility between batches and to be less time consuming[89]. Thus, a total of six Au-nanoprobes sequences were designed to detect each possible allele of the three SNP of interest, in order to characterize homo- and heterozygotes individuals (see Table 3.1).

Table 3.1 - Au-nanoprobes and their fully complementary targets. Synthetic ssDNA targets and plasmids harboring the template for *in vitro/in vivo* transcription.

Au-nanoprobe	Synthetic ssDNA oligonucleotide	Plasmids/ Transcription products
BC1_2731C	sBC1_2731C	pBC1_2731C
BC1_2731T	sBC1_2731T	pBC1_2731T
BC1_3232A	sBC1_3232_38AG	pBC1_3232_38AA
BC1_3232G	sBC1_3232_38GA	pBC1_3232_38GG
BC1_3238G	sBC1_3232_38AG	pBC1_3232_38GG
BC1_3238A	sBC1_3232_38GA	pBC1_3232_38AA

Characterization of Au-nanoprobes

The characterization of Au-nanoprobes' stability upon increasing ionic strength is essential for an efficient target detection by the non-cross-linking colorimetric assay, i.e. the determination of the minimum ionic strength necessary to induce aggregation or the Critical Coagulation Concentration (CCC).

To determine the CCC values of the synthesized Au-nanoprobes, a set of titration assays with the Au-nanoprobes and increasing concentrations of NaCl or $MgCl_2$ were performed and the UV-visible absorption spectrum were registered in order to detect the colorimetric changes associated with AuNPs' coagulation/aggregation.

The differences in stabilization between the AuNPs and Au-nanoprobe for 0.1M NaCl can be observed in Figure 3.4. The added salt revealed to be sufficient to induce a complete

aggregation of the AuNP alone which can be observed by the shift of the SPR band from 525nm to 700nm, but did not induce aggregation of the Au-nanoprobes, which remained with the SPR maximum peak unaltered at 525nm.

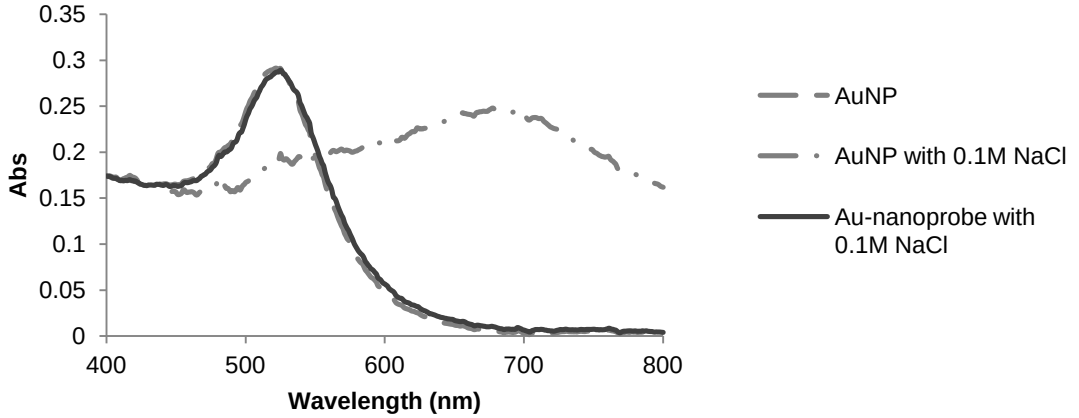

Figure 3.4 – AuNPs vs Au-nanoprobes stability. UV-visible absorption spectrum of the AuNPs and Au-nanoprobes in the presence of NaCl. AuNPs alone (AuNP) and AuNP or Au-nanoprobe in the presence of 0.1 M NaCl (AuNP with 0.1M NaCl and Au-nanoprobe with 0.1 M NaCl, respectively).

Only two nanoprobes (i.e. BC1_2731C and BC1_2731T) aggregated with the addition of 2.5 M NaCl. On the other hand, all Au-nanoprobes aggregated in the presence of ≥40 mM $MgCl_2$. When compared to the AuNP alone, the increased stability of the Au-nanoprobes (e.g. ≥2.5 M for Au-nanoprobes *vs.* <0.1 M NaCl for AuNPs) indicates an efficient functionalization of AuNP with the thiol-modified ssDNA, which is known to increase the AuNP stability to salt induced aggregation[112]. The $MgCl_2$ electrolyte was chosen to induce aggregation in the non-cross-linking assays, since it was the only one capable of inducing aggregation in all Au-nanoprobes. Additionally, the use of NaCl involved the addition of large volumes to the solution, which restricted the capacity of adding other analytes to the assay, such as the target. Aggregation of Au-nanoprobes as function of salt concentration can be easily followed by the red-shift of the SPR peak – Figure 3.5.

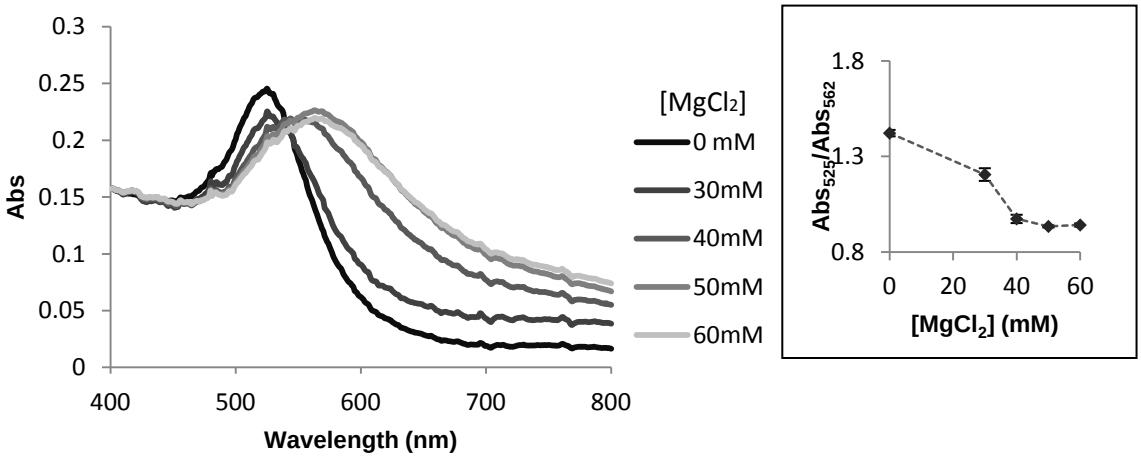

Figure 3.5– Au-nanoprobe stability to increasing salt concentration. UV-visible absorbance spectrum of the BC1_2731T Au-nanoprobe to increasing $MgCl_2$concentrations (0 to 60 mM). Inset: Abs ratio *vs.* $MgCl_2$ concentration. Error bars represent standard deviation of three independent assays.

Differences in stability and capacity of hybridization have been related to Au-nanoprobe density[112, 113]. Thus it was also important to quantify the modified oligonucleotides immobilized to the AuNPs' surface. The quantification of Au-nanoprobe density for each Au-nanoprobe was achieved by fluorescence measurements after the release of the thiol-modified oligonucleotide with DTT.

The CCC values determined for $MgCl_2$ for each probe are shown in Table 3.2 as well as the maximum SPR absorbance peak and the quantification of the thiol-modified oligonucleotides at the AuNP's surface.

Table 3.2- Characterization of Au-nanoprobes. Au-nanoprobes' maximum SPR peaks upon salt aggregation, CCC and oligonucleotides density at AuNPs' surface for all Au-nanoprobes.

Probe	SPR peak upon aggregation (nm)	CCC for $MgCl_2$ (mM)	Au-nanoprobe density (oligo/NP)
BC1_2731C	562	40	64
BC1_2731T	562	50	111
BC1_3232A	605	40	56
BC1_3232G	605	40	81
BC1_3238A	564	40	170
BC1_3238G	564	70	34

The CCC values are similar for all Au-nanoprobes (40 mM $MgCl_2$), except for the BC1_3238G probe (70 mM $MgCl_2$). This difference could be related to differences in Au-nanoprobe density, since higher Au-nanoprobe densities have been reported to confer higher stability to salt

induced aggregation[112]. However, this seems not to be the case since the BC1_3238G probe is the least functionalized but shows the highest CCC value. Moreover, the CCC Au-nanoprobe stability for all the other Au-nanoprobes seems also not to be related to their surface functionalization, i.e. the CCC value remains constant despite the significant differences in their functionalization. Many other factors have been reported to be associated to differences in stability, such as sequence and length of the probe, secondary structures, etc[112]. These could compensate for the differences in surface functionalization and explain the similar CCC values observed for the majority of the Au-nanoprobes.

Besides CCC determination for each probe, Au-nanoprobe characterization allowed to determine the maximum SPR shift of each probe essential to data treatment. The Au-nanoprobe SPR absorption band peaks upon salt addition (SPR of the aggregated form) varied from 562nm to 605nm and were specific to each probe, i.e. they are maintained between different batches of the same Au-nanoprobe, indicating a possible major influence of the probe sequence.

In order to quantify and better assess AuNP/Au-nanoprobe maintenance of dispersion after salt addition, a percentile ratio (%Dispersion) was calculated, see Equation 1. The absorbance ratio (Abs ratio) is obtained by the ratio between the absorbance of the maximum wavelengths characteristic to the dispersed and aggregated form of each Au-nanoprobe.

$$\%\,Dispersion = 100 * \frac{Abs\ Ratio_{\ sample} - Abs\ Ratio_{aggregated\ form}}{Abs\ Ratio_{\ dispersed\ form} - Abs\ Ratio_{aggregated\ form}} \qquad \text{Equation 1.}$$

Hence for each non-cross linking assay the %Dispersion was calculated relatively to a blank assay - Au-nanoprobe alone with salt, which gives the Abs ratio to the maximum aggregated form, and an assay performed without adding any salt, which gives the Abs ratio of the dispersed form. At the CCC, values of %Dispersion near zero are associated with Au-nanoprobes that have aggregated, for which the solution color changes from red to blue. On the other hand, the higher the %Dispersion, the more stable is the Au-nanoprobes system, where 100% of %Dispersion is related to the Au-nanoprobes without salt addition.

To define a threshold between a negative and a positive assay, in this work a negative result was considered to be the %Dispersion that falls in between the signal of the negative control (a non-complementary target) plus three times their standard deviation (Adapted from Zhang *et al.*)[114]. It is well mention that some absorbance shifts can be caused by other factors that are not due to SPR shifts, like precipitation and variations in optical path length (e.g. in microplate reader measurements), these shifts were corrected by performing the normalization at 400nm, once the influenced by SPR changes due to aggregation in this wavelength is minimized.

Target recognition - Synthetic oligonucleotide

The capacity of hybridization of the Au-nanoprobes to a complementary target was first tested using synthetic oligonucleotides as targets. The %Dispersion of each Au-nanoprobes in the presence of their complementary, mismatched and a non-complementary targets is presented in Table 3.3 and as an example the values obtained for BC1_2731C are graphically demonstrated in Figure 3.6.

Table 3.3- Non-cross-linking assay using synthetic ssDNA targets

Au-nanoprobe (2.5nM)	%Dispersion (average of three assays ± STD)		
	Complementary target (1pmol/µL)	Mismatch target (1pmol/µL)	Non-complementary target (1pmol/µL)
BC1_2731C	66 ± 7	66 ± 3	1.7 ± 1.3
BC1_2731T	81 ± 8	77 ± 5	0.2 ± 5
BC1_3232A	49 ± 4	52.1 ± 3.5	3 ± 2
BC1_3232G	98 ± 3	90 ± 6	3 ± 2.5
BC1_3238A	68 ± 1.4	69 ± 0.5	2 ± 0.3
BC1_3238G	34 ± 4	16.6 ± 1.8	3 ± 2.6

All Au-nanoprobes presented a much greater %Dispersion when in the presence of a complementary/mismatch target, while the presence of a non-complementary target did not prevent the Au-nanoprobe aggregation. These results further confirm the efficient functionalization of the AuNP with the thiol-modified oligonucleotides and their capacity to effectively hybridize to the corresponding complementary targets. Furthermore, the differences of %Dispersion observed between Au-nanoprobes designed for the same locus may have a contribution of different levels of functionalization, where a better %Dispersion is achieved for the more functionalized Au-nanoprobes in the presence of their complementary targets (see also Table 3.2)

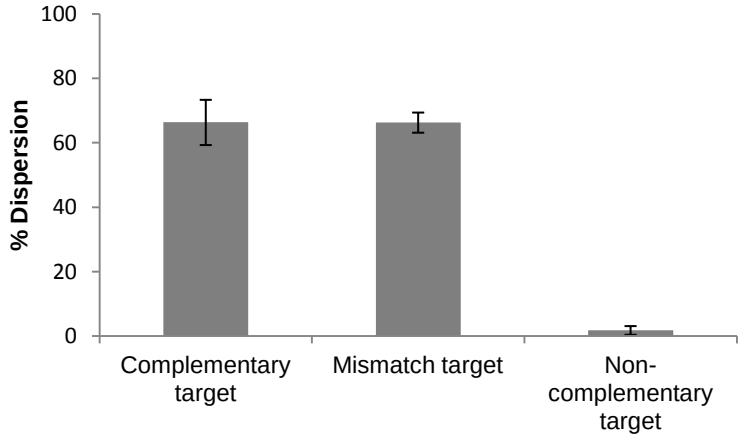

Figure 3.6 - %Dispersion of Au-nanoprobes with ssDNA upon salt addition. As an example, the %Dispersion for theBC1_2731C probe in the presence of the complementary t (sBC1_2731), mismatch (sBC1_2731T) or non-complementary (sBC1_3232AG) target is presented. The targets final concentration was 1 pmol/µL and spectrum data was registered 15 minutes after the addition of 40 mM $MgCl_2$. Error bars represent standard deviation of three independent assays.

Discrimination between complementary and mismatched targets was not possible for all Au-nanoprobes with exception to BC1_3238G probe, for which the discrimination between complementary and mismatch was possible. This incapacity of mismatch discrimination when using ssDNA oligonucleotides has been previously reported, but was overcome when using asymmetric PCR products as targets, suggesting a dependence of the single base resolution on length and complexity of the target[89]. Considering this, the detection of a single mismatch within *in vitro*/*in vivo* transcripts may still be possible, despite the results observed for the ssDNA oligonucleotides.

Limit of detection

The limit of detection of the Au-nanoprobe is also an important parameter to take into account when developing a genotyping method, as well as the determination of the capacity of target quantification. Quantification of different alleles' expression is an important tool in gene expression analysis. Therefore, the non-cross-linking assays were performed in the presence of increasing target concentration up to 0.2 pmol/µL (as an example, see Figure 3.7).

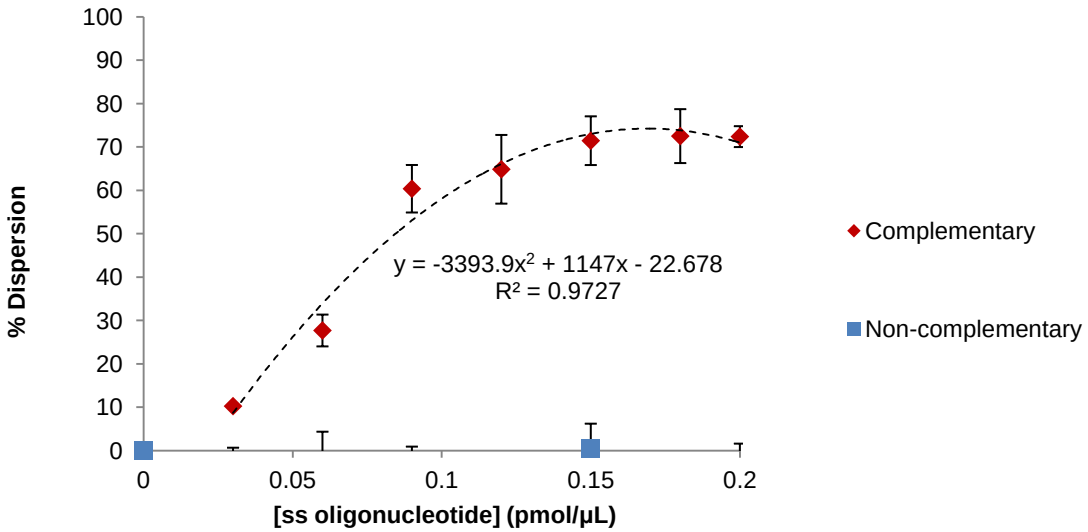

Figure 3.7 - Non-cross-linking target quantification. As an example, the %Dispersion of the BC1_2731C probe in the presence of increasing concentrations of complementary (sBC1_2731C) and non-complementary (sBC1_3232_38AG) ssDNA targets is presented. Spectrum data was registered 15 minutes after the addition of 40 mM $MgCl_2$. Error bars represent standard deviation of three independent assays.

Since the complementary and mismatch synthetic oligonucleotide targets presented the same efficiency of stabilization, these assays were only pursued with complementary and non-complementary targets. Mostly all Au-nanoprobes presented a limit of detection of 0.03 pmol/µl and a linear trend line within the range of 0.03 to 0.12 pmol/µl of complementary target, as one can see in the example of Figure 3.7. Above the 0.12 pmol/µL, the %Dispersion remained unchanged, most likely due to hybridization saturation. For the non-complementary target, the %Dispersion remained negligible for all the tested target concentrations. These results indicate that the Au-nanoprobes are capable of detecting a complementary target and can be used to quantify target concentration until saturation is reached.

Spike-in assays

In order to understand the sensitivity of the Au-nanoprobes in a more complex mixture and try to mimic an assay in total RNA from clinical samples, where mRNA is usually only 5% of the total RNA, a spike-in assay was performed using increasing concentrations of an unrelated total RNA from eukaryotic cells (i.e. *S. cerevisiae*) while maintaining unaltered the concentration of the synthetic oligonucleotide target. The chosen target concentration was the one at which %Dispersion saturation is achieved, i.e. 0.12 pmol/µL. Since the Au-nanoprobes have been

designed to detect *BRCA1* mRNA, which is a low expression gene, the concentration of total RNA chosen were the ones that would make the 0.12 pmol/µL of target present in 1%, 0.7 %, 0.4% and 0.25% of total RNA. To control the influence of the total RNA alone, a blank assay was performed using only total RNA for each concentration. The blank assay was subtracted to the complementary and non-complementary assay (Figure 3.8).

The %Dispersion provided by the complementary target remained unaltered for up to 0.7% of target in total RNA, slightly decreasing for lower target/total RNA percentages (i.e. 0.4 and 0.25%). The total RNA alone revealed to increase the Au-nanoprobes' %Dispersion upon salt addition with increasing concentration of total RNA. Most likely this is due to the presence of a huge amount of single stranded nucleic acids which can adsorb non-specifically to the AuNP surface, thus providing great stabilization against salt induced aggregation [112]. For this reason the %Dispersion for 0.4 and 0.25% of complementary target decreases and this may also be the reason to the increase in %Dispersion observed for the non-complementary target (behavior more pronounced in the BC1_2731T probe (Figure 3.8 B). The decrease of % Dispersion observed for the complementary oligonucleotide may also be due to the increase of entropy of the system which can hamper the hybridization of complementary targets to the Au-nanoprobes. Nonetheless, the discrimination between complementary and non-complementary target was possible for all the tested target/total RNA ratios.

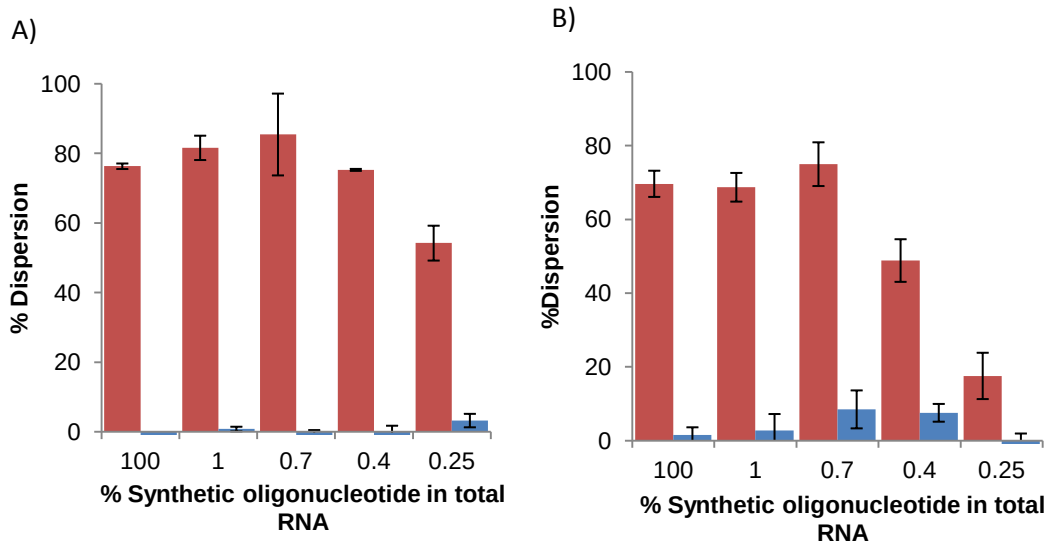

Figure 3.8 - **RNA spike-in assay**. %Dispersion of Au-nanoprobe (BC1_2731C probe (A) and BC1_2731T probe (B) with decrease % of complementary (red bars) and non-complementary (blue bars) targets in total RNA, 15 minutes after addition of 40 mM $MgCl_2$. The target final concentration was 0.12 pmol/µL spiked-in with increasing concentrations of *S. cerevisiae* total RNA. Error bars represent standard deviation of three independent assays.

3.3 *in vitro* RNA transcript detection

Once the probes have been thoroughly characterized and calibrated, their capacity for *in vitro* transcription detection was tested.

As mentioned before, the direct use of *in vitro* transcription cannot be applied in the non-cross-linking detection assays since the product of transcription induces aggregation of Au-nanoprobes (Figure 3.9). The SPR absorbance peak of the assays with complementary and non-complementary targets is centered at 730nm. This is an enormous shift of the SPR absorbance peak when compared to the blank assay, suggesting that something in the transcription product promotes a further Au-nanoprobe aggregation to the level of the unmodified AuNP. One hypothesis could be the reduction of the thiol-modified oligonucleotides by the presence of DTT which could remove the oligonucleotides from the NPs surface, leading to a bigger SPR shift[112].

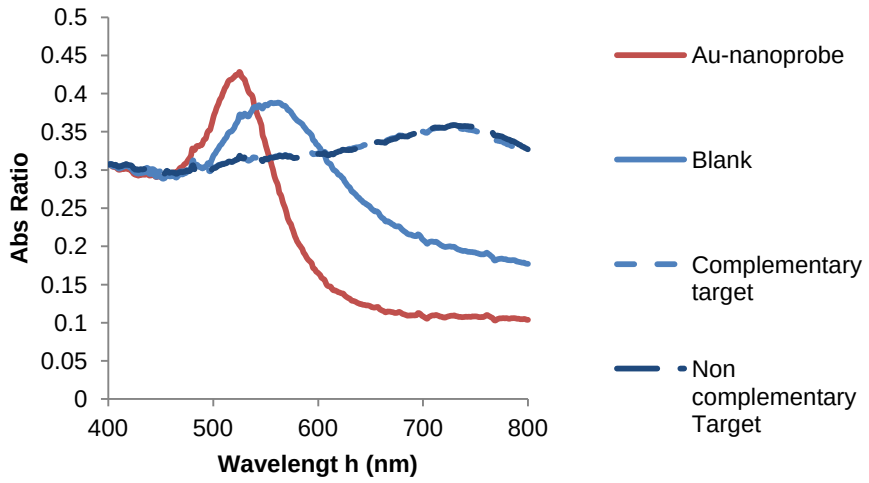

Figure 3.9- Non-cross-linking assay with unpurified *in vitro* transcripts. UV-visible absorbance spectrum of BC1_2731C probe without salt (Au-nanoprobe) and salt addition (Blank), and in the presence of unpurified complementary (complementary target) and non-complementary (non-complementary target) *in vitro* transcripts. The final concentration of target was 0.1 pmol/μL and spectrum data was registered 15 minutes after the addition of 40 mM $MgCl_2$ (except for "Au-nanoprobe" where $MgCl_2$ was substituted by 10mM phosphate buffer (pH8)).

To try to circumvent the problem mentioned before and following the purification problems mentioned in **3.1. Biological Sample preparation,** two purification techniques were approached in parallel. The NAP-5 approach for purification of the *in vitro* transcript did not prove to be effective. In this case, both complementary and non-complementary targets

revealed to stabilize the Au-nanoprobes upon salt addition (Figure 3.10). Once the NAP-5 column does not exclude enzymes, one possibility of this strong non-specific stabilization can be due to the presence of T7 RNA polymerase which can adsorb to the AuNPs' surface, similarly to what occurs to other proteins [115,116,117]. On the other hand, when purifying the *in vitro* transcript through the phenol/chloroform extraction (which enables the exclusion of the T7 RNA polymerase) the discrimination between complementary and non-complementary mRNA targets has been finally achieved (Figure 3.10). Hence, the phenol/chloroform extraction was the method used for the subsequent *in vitro* transcripts purification.

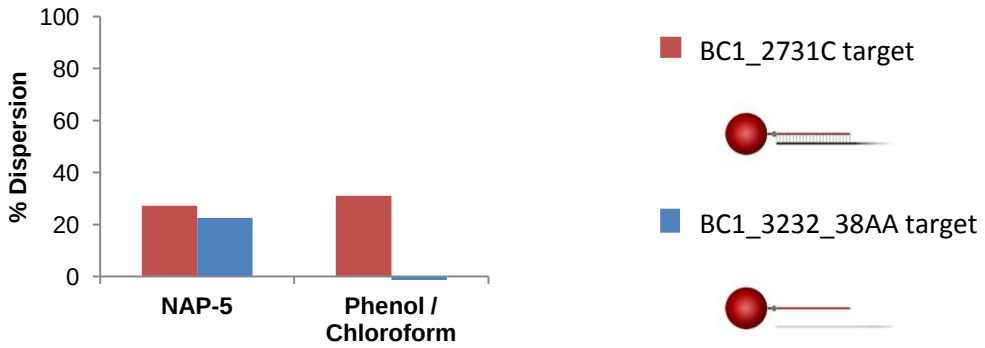

Figure 3.10 - Effect of transcript purification on the non-cross-linking method. Colorimetric assays with BC1_2731C probe in the presence of a complementary (BC1_2731C) and non-complementary (BC1_3232A) *in vitro* transcripts purified by two different techniques: NAP-5 and Phenol/ Chloroform extraction. The final concentration of target was 0.1 pmol/µL and spectrum data was registered 15 minutes after the addition of 40 mM $MgCl_2$.

The mRNA transcripts were quantified through agarose gel electrophoresis since the phenol/chloroform purification does not rule out the presence of the template DNA and DNA/RNA hybrids, which can interfere with transcript quantification through UV/visible spectroscopy. Therefore, the amount of RNA was estimated by comparison between the fragment intensity of the sample and the nearest fragment of the RNA ladder (i.e. 500 b). The RNA from the DNA/RNA hybrid and the DNA template present in the transcription product may also hybridize to the Au-nanoprobes and, thus, stabilize them against salt induced aggregation, interfering with the RNA assays. However, the DNA/RNA hybrid could not be quantified due to the lack of a corresponding ladder or a reference sample with a known concentration that would enable quantification of such molecules. Nevertheless, the presence of such RNA does not present an issue, since disregarding the contribution of such RNA is only going to affect the assessment of the assay sensitivity to mRNA and not its capability for SNP detection in RNA. On the other hand, the presence of the dsDNA template is believed not to interfere with hybridization, since the template concentration is below the limit of detection described for the

non-cross-linking assay with DNA samples (i.e. 900 ng of total DNA)[90,91]. Even though, a parallel assay was performed in order to check if the template could provide stabilization to the Au-nanoprobes against salt induced aggregation. The concentration of template used was 600ng, which exceeded the concentration of DNA template present in the RNA detection assays and still no stabilization of the Au-nanoprobes to salt induced aggregation was observed. Therefore, we can exclude the contribution of the template to the stabilization of the Au-nanoprobes and, hence, only the remaining RNA can eventually provide such stabilization.

Finally, having defined the purification method for the *in vitro* transcripts, the non-cross-linking assay was tested for the single base resolution of the non-cross-linking in RNA molecules, using *in vitro* RNA transcripts. For this purpose, 0.03, 0.06 and 0.08 pmol/μL of transcript were used, as shown in Figure 3.11.

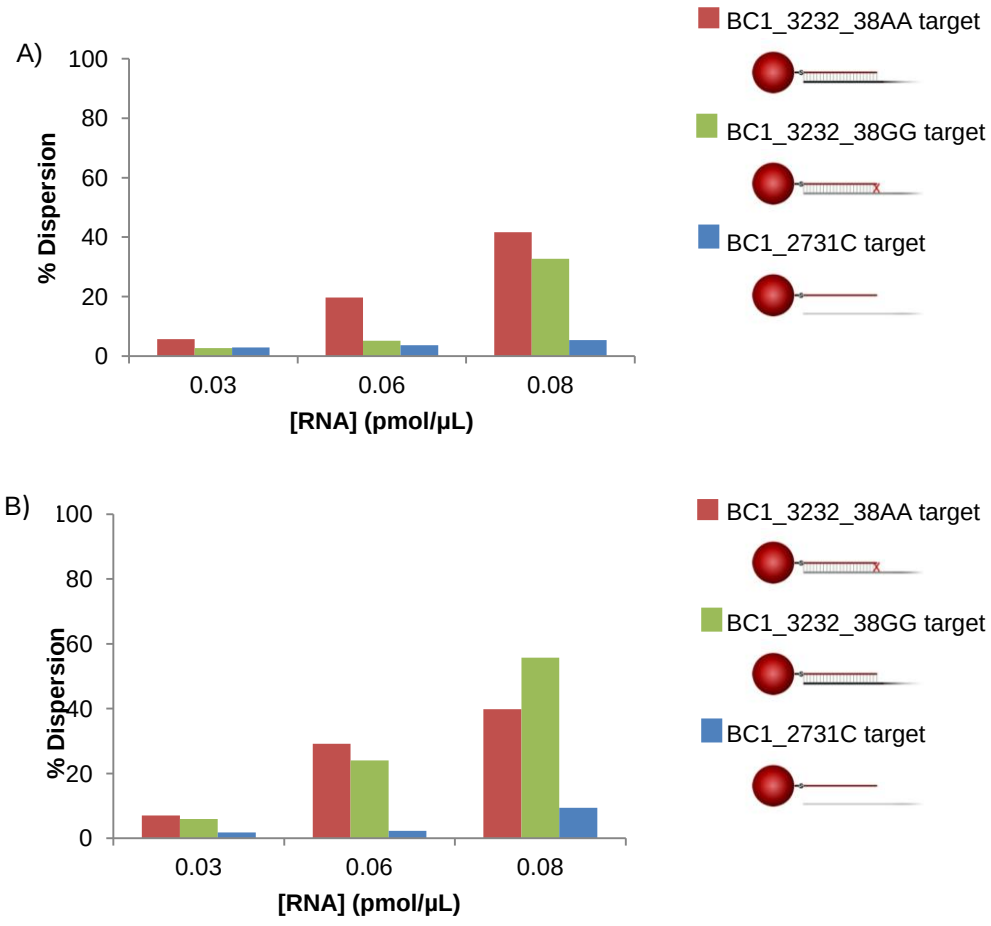

Figure 3.11 - SNP detection assay with *in vitro* transcripts. BC1_3232A probe (A) and BC1_3232G probe (B) in the presence of 0,03, 0.06 and 0.08 pmol/μL of complementary (BC1_3232_38AA and BC1_3232_38GG, respectively), mismatch (BC1_3232_38GG and BC1_3232_38AA, respectively) and non-complementary (BC1_2731C) in vitro transcript targets. Spectrum data was registered 15 minutes after the addition of 40 mM $MgCl_2$.

In the presence of 0.03 pmol/μL, the discrimination was not possible probably due to sensitivity issues, although, at these minimum concentrations one can see differences between complementary and non-complementary targets. This behavior is enhanced with the increase in target concentration, i.e. 0.06 pmol/μL. At this target concentration, the BC1_3232A probe appears to have better sensibility than the BC1_3232G probe to discriminate between complementary and mismatch target. However, BC1_3232G probe did not seem to be able to discriminate the single base mismatch until saturation is reached at 0.08 pmol/μL, where differences of %Dispersion due to differences in target quantification no longer take effect, i.e. the Au-nanoprobes' %Dispersion can vary for different target concentrations up to saturation, above which the %Dispersion no longer varies. In these assays, both BC1_3232A and BC1_3232G probes were able to discriminate the corresponding single base mismatch directly in the RNA target when using 0.08 pmol/μL of transcript. Although the mismatch demonstrates an increase %Dispersion when compared to the non-complementary target, a difference of 10% between complementary and mismatch is observed for the BC1_3232A probe and 15% for the BC1_3232G probe. This mismatch discrimination may be further enhanced by optimizing hybridization conditions, in order to reduce the stabilization induced by the mismatch target at 0.08 pmol/μL.

It can also be observed the increase %Dispersion caused by increased concentrations of the non-complementary target, although this target clearly did not prove to stabilize the probes. This slight increase in stabilization can be due to presence of more nucleic acids that are responsible for an increase in the entropy of the system resulting in more stabilization[78].

It has been recently reported the influence of functionalization in capacity of mismatch discrimination[88]. Although these Au-nanoprobes' densities are in accordance to the best capacity of discrimination (80 oligo/NP), it is worth mentioning that these studies were performed with 40-mer DNA targets, while the RNA targets used in the present report are around 400 bases long. The BC1_3232A is less functionalized than BC1_3232G (56 oligo/NP and 81 oligo/NP, respectively) and have, without optimization, a better discrimination capacity between complementary and non-complementary targets. Even though optimization was not carried out, due to material and time constraints, it was possible to demonstrate as proof-of-concept the discrimination of SNP in RNA using the non-cross-linking method. In addition, with the increase of complementary target concentration there is an increase in stability of the Au-nanoprobes system indicating also the potential of quantification of the system for each allele.

3.4. *In vivo* RNA transcript detection

The plasmids harboring fragments of interest were transformed in *E.coli* BL21 (DE3) pLysE cells in order to induce *in vivo* transcription by IPTG. The BC1_3232A probe was the first tested with

in vivo transcripts, since it presented the best capacity of detection when using *in vitro* RNA. Before checking the possibility of single mismatch resolution, the first attempt was to verify the capacity of hybridization with the complementary target. Thus, *E.coli* BL21 harboring the plasmid pBC1_3232A_38A was submitted to induction with IPTG. As negative control, a *E.coli* BL21 transformed with pUC19 was used. Additionally, the expression induced with IPTG was controlled by an assay without adding IPTG. Total RNA extraction was performed after the induction of expression by IPTG. The total RNA integrity was confirmed in agarose gel (Appendix E). The ratio Abs260nm/Abs230nm was below 1 indicating contaminations with some impurities (e.g. phenol). Nonetheless, the non-cross-linking assay was performed using 0.5 µg of total RNA. This value was chosen since it was the upper value used in the RNA detection reported by Baptista *et al*.[78]. All total RNA samples provided stabilization of the Au-nanoprobes system, indicating that the impurities present within the purified transcript are most likely hampering the colorimetric assay. One hypothesis is the presence of proteins that may adsorb to AuNPs and hamper aggregation upon salt addition, as discussed previously[115,116,117]. Hence, future optimization of the total RNA extraction is needed in order to achieve samples suitable to conduct the non-cross-linking assays with *in vivo* transcripts.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The present work was developed with the purpose of verifying if the non-cross-linking method would allow to detect single base mismatches in RNA molecules. Therefore DNA biological samples gently provided by the IPO, harboring three of SNPs in *BRCA1* gene associated with breast cancer, were cloned in an expression vector for further *in vitro* transcription. The purification of the transcript revealed to be a limitation in this study, not only because of the low yield of the purification step but also due to the sensitivity of the Au-nanoprobes system to dielectric changes of the medium. The phenol/chloroform extraction was the only method tested for the purification of *in vitro* transcriptions that revealed to be efficient, allowing to carry out the needed non-cross-linking assays. A setback associated with the choice of this method is the necessity of quantification of the transcript present in the samples by agarose gel, which is a more cumbersome technique when compared to the quantification through UV-visible spectroscopy.

The Au-nanoprobes designed for detection of the RNA fragments were characterized in terms of stability, capacity of hybridization and their detection limit. Mostly all Au-nanoprobes showed a limit of detection of 0.03 pmol/μL, with maximum discrimination between complementary and non-complementary targets attained at 0.12 pmol/μL of synthetic ssDNA. Moreover, the synthesized Au-nanoprobes presented a linear variation of their %Dispersion for complementary targets up to 0.12 pmol/μL, which allows for target quantification.
In order to mimic the complexity of real biological samples, a spike-in assay using total RNA from *S. cerevisiae* was performed. The detection of a complementary target was possible up to a ratio of 0.25% target/total RNA, which resembles the percentages of RNA expression found in real biological samples. The detection of SNP within *in vitro* transcripts was possible for the BC1_3232A and BC1_3232G probes when using 0.08 pmol/μL of RNA. For the other Au-nanoprobes, the detection of their respective SNPs was inconclusive and may require further optimization. For this reason, it is important to perform more assays in order to determine the universal capabilities of the non-cross-linking system for SNP detection in RNA. Optimization may be achieved through a more homogenous functionalization of the Au-nanoprobes to a level where single base mismatch resolution is optimized. Moreover, hybridization conditions and comprehensive studies about the kinetics of aggregation could allow to optimize SNP discrimination by the non-cross-linking method. Once the mismatch discrimination has been optimized, the capacity of allele quantification should be possible by performing the non-cross-linking assay with differential concentrations of complementary and mismatch targets. In a near future optimization of the *in vivo* transcripts detection can also be performed specially using the Au-nanoprobes that thus far allowed to discriminate SNP within *in vitro* transcripts, i.e. BC1_3232A and BC1_3232G probes.

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APPENDICES

Appendix A - Products of PCR amplification obtained by different set of primers

Table A.1 - Products of PCR amplification obtained by different set of primers.

	Size of the amplicon (pb)		
Plasmid	**Primers Fw and Rev specific to the insert**	**Primers pJET1.2 Fw and Rev specific to the insert**	**Primers pJET 1 and primer Rev of the insert**
pBC1_2731C pBC1_2731T	381	444	473
pBC1_3232_38AA pBC1_3232_38GG	394	457	486

Appendix B - pJET1.2/blunt vector map.

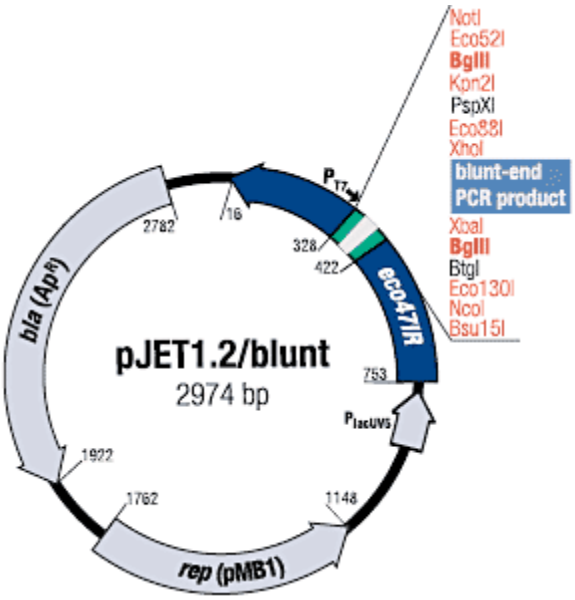

Figure A.1. pJET1.2/blunt vector map.

Appendix C - Sequences of the plasmids

plasmid pBC1_2731C

```
   1 GCCCCTGCAG CCGAATTATA TTATTTTTGC CAAATAATTT TTAACAAAAG
  51 CTCTGAAGTC TTCTTCATTT AAATTCTTAG ATGATACTTC ATCTGGAAAA
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Appendix D - Isolation of *in vitro* RNA transcripts by agarose gel purification

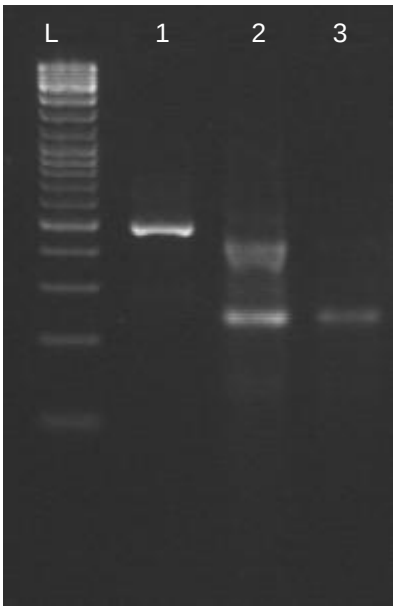

Figure A.2. Agarose gel electrophoresis of transcripts after purification by freeze and squeeze method. Electrophoresis (80 V, 1 h30) in agarose gel (2%, 1x TBE, 0.5x Gelred ^{TM}). L) GeneRuler™ DNA Ladder Mix, ready-to-use 1) BC1_2731C template (473 pb); 2) BC1_2731C in vitro transcription (431b); 3) purified BC1_2731C in vitro transcription (431b).

Appendix E - Extraction of total RNA from *E.coli* BL21 clones

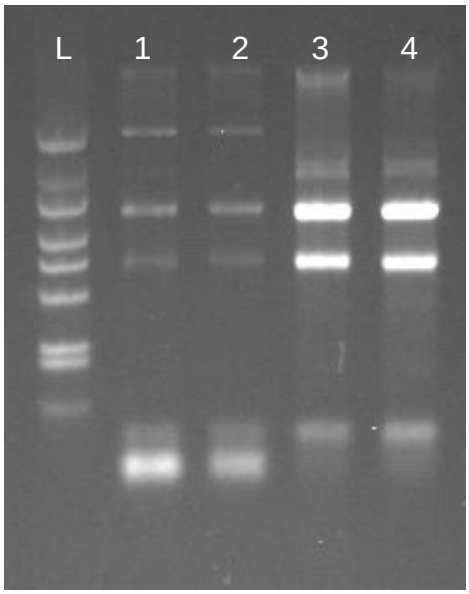

Figure A.3. Agarose gel electrophoresis of Total RNA extracted from *E.coli* BL21 clones. Electrophoresis (90 V, 1 hour) in agarose gel (2%, 1x TBE, 0.5x Gelred ™). L) RiboRuler™High Range RNA Ladder. 1) pBC1_3232A_38A induced with IPTG, 2) pBC1_3232A_38A without IPTG induction 3) pUC19 induced with IPTG 4) pUC19 without IPTG induction.